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Prenatal and postnatal energetic conditions and sex steroids levels across the first year of life

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Abstract

Objectives—Human biologists have documented variability in reproductive maturation, fertility, and cancer risk related to developmental conditions. Yet no previous studies have directly examined the impact of pre- and post-natal energetic environments on sex steroids in infancy, a critical period for hypothalamic-pituitary-gonadal axis development. Thus, we examined the impact of maternal characteristics, birth size, and feeding practices on fecal sex steroid production in a longitudinal sample of 31 American infants followed from 2 weeks to 12 months of age.

Methods—Maternal characteristics and birth size were collected at study enrollment, infant diet was assessed through weekly 24-hr food diaries, and anthropometrics were measured weekly. Fecal estradiol and testosterone levels were assessed weekly using validated microassay RIA techniques. Mixed models were used to test for associations between maternal and birth characteristics, feeding practices, and sex steroids across the first year of life. Formal mediation analysis examined whether the relationship between infant feeding and hormone levels was mediated by infant size.

Results—Maternal and birth characteristics had persistent effects on fecal sex steroid levels, with taller maternal height and larger birth size associated with lower estradiol levels in girls and higher testosterone levels in boys. Infant diet was also associated with sex steroid levels independently of infant size. Formula feeding was associated with higher estradiol levels in boys and girls and with higher testosterone in girls.

Conclusion—These results suggest that markers of early energy availability influence sex hormone levels with potential long-term consequences for reproductive development and function.

Keywords

estradiol; testosterone; infancy; infant feeding; birthweight

Anthropological investigations of reproductive function have long established the importance of developmental conditions in shaping maturational tempo and post-pubertal sex steroid levels (Campbell and Leslie 1995; Ellison et al. 1993; Vitzthum et al. 2002). More recently, research has documented long-term impacts of gestational and perinatal environments on the reproductive system, ranging from the timing of puberty to post-pubertal fecundity and the risk of reproductive cancers in later life. Experimental evidence from animal models documents accelerated pubertal timing among offspring of both maternal restricted and maternal high fat diets (Sloboda et al. 2009). Observational human studies report associations between nutrition *in utero* and the timing of menarche (Cooper et al. 1996) and adult fertility (Painter et al. 2008). These data focus attention on the influence of nutrient and endocrine signals, received both pre- and post-natally, on pubertal timing and adult reproductive function. As critical components of reproductive success, reproductive development and fecundity may be particularly sensitive to energetic cues, such as maternal nutritional status, hormonal exposure during pregnancy, and post-natal nutrition during early development (Gluckman and Beedle 2007; Sloboda et al. 2011). How these cues translate early life experience to reproductive biology remains largely unknown.

More specifically, the impact of pre- and postnatal conditions on the tempo of reproductive development and adult reproductive function appears to be opposing. A number of studies report associations between poor prenatal conditions and more rapid sexual development. For example, low birth weight has been associated with earlier sexual maturation (Adair 2001; Karaolis-Danckert et al. 2009; Persson et al. 1999; Terry et al. 2009) and elevated sex steroids in adolescence and adulthood in various samples (Finstad et al. 2009; Opdahl et al. 2008; Vanbillemont et al. 2010), though a positive relationship was reported between adult female estradiol levels and birth ponderal index by Jasienska and colleagues (Jasienska et al. 2006a; Jasienska et al. 2006b). In contrast, it is good energetic postnatal environments that are associated with rapid growth during infancy and childhood, earlier ages at maturation, and elevated adult sex steroid levels (Karaolis-Danckert et al. 2009; Kuzawa et al. 2010; Ong et al. 2009; Terry et al. 2009). Energetically poor postnatal environments, assessed through slow growth rates or morbidity from infectious disease, have been associated with delayed maturation and lower adult hormone levels (Khan et al. 1996; Nunez-de la Mora et al. 2007; Nunez-de la Mora et al. 2008). These contrasting influences of pre- and post-natal energy availability on maturational tempo have led some researchers to propose the existence of two different maturational signals driving sexual maturation: a prenatal deprivation signal that promotes early reproduction in the face of a dangerous, energy-poor world and a postnatal signal of energy sufficiency to support earlier reproduction (Gluckman et al. 2007).

The importance of birth size and infant and child growth on long-term reproductive function suggests that the reproductive axis is sensitive to ecological conditions both *in utero* and postnatally. The mechanisms linking prenatal and postnatal nutritional status to these later reproductive outcomes, however, remain unknown. Candidate hormones include the sex steroids, acting through early organizational effects on the hypothalamic-gonadal (HPG) axis. Cross-sectional studies of neonatal hormone levels (Ibanez et al. 2002) suggest an association between birthweight and sex hormone levels during early infancy. Cross-

sectional and limited longitudinal studies have documented an early postnatal rise in gonadotropins, which stimulates gonadal testosterone and estradiol production at levels within the low end of the adult range (Bergada et al. 1999; Forest and Ducharme 1993). In addition to influencing brain and behavioral development (Alexander et al. 2009), this postnatal HPG activation plays an important role in the development of the reproductive system. Clinical studies in hypogonadal boys (Main et al. 2000; Main et al. 2006) and experimental primate models (Mann and Fraser 1996) document that postnatal testosterone production is necessary for normal genital development, Sertoli cell proliferation, and germ cell differentiation. Similarly, estradiol levels in neonatal girls are associated with ovarian size, follicular differentiation, and breast tissue mass (Schmidt et al 2002). Energetic and ecological conditions influencing gonadal development *in utero* and sex steroid production in infancy, then, could shape long-term hormonal variability and reproductive function during a critical period for the organization of the hypothalamic-gonadal axis (Boas et al. 2006; Forest and Ducharme 1993; Main et al. 2006; Schmidt et al. 2002).

Evidence that sex steroid levels play an important role in early reproductive development taken together with the well-documented association between early development and adult reproductive function justify a closer examination of sex steroids in infancy. This is the first study to examine the impact of early energetic environments on infant sex steroid levels. We utilize novel, non-invasive measures of fecal sex steroids to examine the association between pre- and post-natal energetic environments and sex steroid production during infancy. Based on the contrasting effects of pre- and post-natal energy sufficiency on tempo and quality of reproductive development (Gluckman et al. 2007), we hypothesize that maternal characteristics and larger birth size, indicators of nutritional sufficiency *in utero*, are associated with lower fecal sex steroids across the first year of life. Conversely, we hypothesize that formula and solid feeding, which are associated with higher energy intake (Heinig et al. 1993) and more rapid infant growth over the first year of life (Butte et al. 2000b; Dewey 2009), are associated with higher steroid levels. Since feeding practices are associated with differences in infant size and breast- and formula-feeding may differentially influence linear growth and body composition (Thompson 2012), we also test whether breast-, formula- and solid feeding are independent predictors of sex steroids, controlling for their indirect effects through differential infant growth.

Sample

Thirty-one infants (15 male, 16 female) participated in a prospective, mixed-longitudinal growth study investigating hormonal development during the first year of life. Subjects were recruited from a university-affiliated daycare center, mothers' groups, and lactation support groups. Infants were included in the study if there was no history of gestational diabetes or smoking during pregnancy. All participating infants were born after 37 gestational weeks with birthweights >2500gm and were free of endocrine, metabolic or other clinical conditions known to affect growth or hormonal development during the course of the study. Infants entered the study between the ages of 7 days and 10.5 months (median age at entry 19.6 weeks, IQR = 10.8–32.8 weeks) and were followed weekly for a median of 16 weeks (IQR=16–20.8 weeks). Recruitment and data collection protocols were approved by the Emory University Institutional Review Board.

Infant Feeding

Parents recorded all infant feeding episodes including the types and quantities of foods and liquids consumed in the 24-hours preceding the growth measurement and diaper collection. Parents were instructed on how to fill-out pre-printed diet history forms upon enrollment in the study and were provided with sample forms to guide their entries. Incomplete entries with missing or improbable intakes were checked with the parents and daycare providers.

Infant feeding status was defined on a weekly-basis using these 24-hour food diaries. Feeding type were classified according to the WHO schema (WHO 2001) into 7 categories: 1) exclusively breastfed (EBF) if the infant only received breastmilk and small amounts of medicine or vitamins, 2) exclusively formula fed (EFF) if the infant received formula and no other liquids or solids, 3) mixed fed (MF) if the infant received formula and breastmilk but no other liquids or solids foods, 4) complementary fed (CF) if the infant received breastmilk and supplementary solids and liquids (not including non-human milk), 5) complementary formula fed (CFF) if the infant received formula and other foods and liquids (except breastmilk), 6) complementary mixed fed (CMF) if the infant received breastmilk, formula and other solid foods and liquids; and 7) weaned (W) if the infant received no formula or breastmilk. Only 1 weekly observation fell in to category 7, so this category was excluded from analysis, resulting in a final 6-level categorical variable. Since feeding type was strongly associated with infant age, this variable was stratified by solid feeding status into 2 categorical variables: one comparing formula feeding and mixed feeding to exclusive breastfeeding and the other comparing complementary formula feeding and complementary mixed feeding to complementary feeding.

Anthropometry

Infant 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 using the maximal stretch technique by two observers (Lampl et al. 2001) to the nearest 0.10 cm using an infant measuring board equipped with fixed headboard and a mobile footboard (Precision Enterprises; Portage, MI). Length was measured in duplicate in the majority (~80%) of measurements, unless the state of the infant precluded additional measurement, and the mean of duplicate measures was used in analysis. Infants' weight was measured using a portable, digital scale accurate to the nearest 10gm, calibrated before measurement (MedWeigh, MS-2410). Infants were weighed either nude or clad in a clean diaper, in which case diaper weight was subtracted from the weight measurement. Subcutaneous skinfolds (triceps, sub-scapular, abdominal, suprailiac, quadriceps and calf) were measured with Holtain skinfold calipers to the nearest 0.1mm and these sites were summed (sumSF) Infant BMI (kg/m^2) was calculated from length and weight measures. Infant BMI was chosen as a measure of overall adiposity based on recent evidence that BMI is more highly correlated with measured %body fat than is ponderal index (Ellis 2010).

Maternal anthropometry prior to and during pregnancy and infant birth characteristics were self-reported and provided from birth records, respectively.

Fecal Sample Collection and Hormone Analysis

Parents were asked to retain soiled diapers after each diaper change during the 24-hour period prior to weekly growth measurements. Soiled diapers were placed in individual zip-top bags, labeled with the date and time of collection and stored in portable coolers chilled with freezer blocks frozen to -80°C . Upon collection, the samples were stored at -80°C until processed.

Fecal samples were excised from the diapers, extracted using methanol, and filtered through microfilter centrifuge tubes (Centrex, Whatman Laboratory). Extracted samples were assayed following validated protocols developed for measuring fecal estradiol and testosterone in human infants (Thompson et al. 2010; Thompson et al. 2011) from modified commercially-available kits (DSL, Texas). All samples were assayed in duplicate and the mean value of duplicate samples was used for analysis. Intra- and inter-assay precision for each assay was within acceptable range for RIAs. Intra-assay precision was 8.09% (mean: 39.34 pg/ml) for the pooled fecal testosterone control and 5.8% (mean: 3.4 pg/ml) and 2.2% (mean: 8.7 pg/ml) for low and high fecal estradiol controls, respectively. Inter-assay precision was 10.8 (mean: 21.86 pg/ml) and 15.39 (mean: 21.3 pg/ml) for male and female fecal testosterone controls, respectively, and 18.5 (mean: 3.3 pg/ml) and 9.9 (mean: 34.3 pg/ml) for low and high fecal estradiol controls, respectively. Assay values were standardized for fecal weight and values are expressed as pg/gm. This protocol yielded an analytic sample of 463 diapers matched to weekly anthropometric measures over the first 12 months.

Data Analysis

Estradiol and testosterone values were not normally distributed and were transformed for regression analyses by taking the natural log of testosterone and the natural log of the estradiol +0.039, identified as the transformation best approximating normality. These transformations normalized the variable distributions (Shapiro-Wilk tests of normality, $p>0.05$ for both hormones). Estradiol values below the detection limit ($n=11$) were expressed as 0 pg/gm. Analyses were repeated with undetectable values set at the limit of detection; this did not alter the results. Repeated-measures mixed model regression was used to iteratively test the association between maternal and birth characteristics and sex steroid levels across the first year of life, controlling for infant age and, in the case of birth size, current size. Subject was included as a random effect in all models to control for subject-specific effects. This approach takes advantage of the repeated growth measures, estimating the mean effect size from within and between-individual variation, providing greater statistical power than standard regression models and accounting for within-subject correlation (Goldstein 2010). All models were stratified by sex.

Our analytic strategy for measuring the association between infant feeding and hormone levels is shown in Figure 1. In the first step, mixed model analysis was used to test the main effect of the two feeding category variables on hormone levels, controlling for age and individual random effects. Effect modification by age was examined through testing interaction terms with likelihood ratio tests, and an interaction term between feeding

category and age was retained when indicated by likelihood ratio testing. Maternal and birth characteristics were not associated with feeding practices and were not included as confounders in these models.

In the second step, we examined whether the association between infant feeding and hormone levels was confounded by infant size, following the mediation procedure recommended by Baron and Kenny (1986). We tested whether the inclusion of infant size measures (length, weight, BMI, and sum of skinfolds) mediated the effect of feeding on estradiol or testosterone levels. After establishing that the predictor (infant feeding) was associated with the outcome (estradiol or testosterone) in step one, we next tested whether the predictor (infant feeding) was associated with the mediator (infant size measure), controlling for infant age and, if indicated by likelihood ratio testing, the interaction between feeding and age. Finally, we tested whether the mediator (infant size) was associated with the outcome (estradiol/testosterone), controlling for infant age and birth size and, if significant, an interaction term between infant feeding and age.

For models that met these 3 requirements – 1) infant feeding was significantly associated with estradiol or testosterone in the absence of infant size, 2) feeding type predicted size, and 3) size significantly predicted estradiol or testosterone level-- we tested whether the inclusion of infant size in the model partially or fully attenuated the association between the feeding type and estradiol or testosterone levels. As a more stringent test of mediation, the Krull and MacKinnon (2001) bootstrapping for multilevel models was used to test whether an association between feeding type and estradiol or testosterone level resulted from the indirect effect of feeding type on infant size. This procedure requires identical covariates along model paths. Consequently, models control only for age and clustering by individual and results are more conservative than those of the mediation analysis.

STATA/SE version 11 (Stata Corporation, College Station) was used for all analyses. Statistical significance was defined as $p < 0.05$.

Results

Sample Descriptives

Maternal and infant characteristics are shown in Table 1. There were few differences in maternal characteristics or infant feeding practices between boys and girls. Girls were marginally more likely to be first-borns and to be born at later gestational age ($p = 0.08$ for both); boys were longer than girls at birth.

Breastfeeding initiation was 100% and breastfeeding was not completed in all infants by the end of the study. Using infant age at study completion in place of age at weaning for these infants resulted in a minimum median duration of breastfeeding of 7.5 months (range: 2 weeks – 12 months). At one month of age, 41% of infants were exclusively breastfed; 27% exclusively breastfed to 6 months. Sixty percent of infants received formula either solely or in addition to breastmilk, with a median age at introduction of 5 months (range: birth-11 months). Sixty-two percent of infants received solids before 6 months of age, and the median age of solid introduction was 5.25 months (range: 4.2–6.75 months).

Maternal and birth characteristics and hormones

Maternal and birth characteristics were associated with infant estradiol and testosterone (Table 2). Estradiol was inversely associated with maternal height, birth length and birthweight, and positively associated with primiparity in girls. Controlling for potential confounding by current infant size, birth length remained a significant independent predictor of estradiol when current length was added to the model ($\beta=-0.08$, $p=0.04$) while the inclusion of current weight attenuated effects of birthweight ($\beta=-0.28$, $p=0.31$). Among boys, later gestational age at birth was associated with higher estradiol and testosterone levels. Testosterone levels were positively associated with BMI at birth in boys. BMI at birth remained a significant predictor of testosterone across the first year when controlling for current BMI ($\beta=0.25$, $p=0.02$). No maternal characteristics were associated with testosterone in either sex.

Infant feeding and hormones

Table 3 shows the mixed model results for the main effect of infant feeding on concurrent measures of infant estradiol and testosterone, controlling for infant age (Figure 1a: Path c). Overall, formula consumption was associated with altered hormone levels in boys and girls, compared to breastfeeding. Among girls, the addition of formula was associated with higher estradiol and testosterone levels compared to breastfeeding alone, both before and after the introduction of solids. Among boys, estradiol levels were higher among exclusively formula fed compared to exclusively breastfed boys and among complementary mixed fed boys in comparison to boys receiving breastmilk and solids. Testosterone levels, however, were lower among boys receiving formula and solids compared to those receiving breast milk and solids.

Mediation analysis—Formal mediation analysis was used to assess whether the relationship between infant feeding and hormones (Figure 1a, path c) was mediated by feeding-related differences in body size and composition (Figure 1b: path a) which in turn determined hormone levels (Figure 1b: path b). These causal pathways between infant feeding and hormone levels were investigated to rule-out an association mediated by infant size. We describe our results for each of these paths below.

Infant feeding and infant size (Figure 1b: Path a): Table 4 shows the results of sex-stratified models testing the main effect of infant feeding on the potential mediator, infant size. In general, breastfed infants were larger than those receiving formula. Compared to exclusively breastfed girls, exclusively formula fed girls were significantly shorter and had lower BMI, while those receiving both breast milk and formula had lower sum of skinfolds. In contrast, among girls receiving solids, girls fed both breast milk and formula were longer and heavier than girls receiving only breast milk and solids. Among boys, combined breastmilk and formula feeding was associated with lower sum of skinfolds than exclusive breastfeeding. After the introduction of solids, boys receiving formula and solids had lower sum of skinfolds than complementary fed boys while boys receiving formula, breastmilk and solids had lower sum of skinfolds and were also shorter and lighter than complementary fed boys.

Infant size and hormone levels (Figure 1b: Path b): Table 5 shows the relationship between the mediator, infant size, and the outcome, hormone levels, stratified by sex and solid feeding. Overall, infant size was more strongly associated with estradiol levels than with testosterone levels. Prior to the introduction of solids, significantly higher estradiol levels were seen among shorter, lighter boys and leaner girls. Similarly, after the introduction of solids, boys with higher estradiol were lighter and leaner. Size showed fewer associations with testosterone. Testosterone was lower among lighter boys and among those with lower BMI. No relationships were found between size and testosterone among girls.

Did infant size mediate the feeding effects on hormones?—To test for mediation, we identified: (1) feeding patterns that were significantly associated with hormone levels (Table 3), (2) those feeding patterns which were also significantly associated with size (Table 4), and (3) those size variables that significantly predicted hormone levels (Table 5). For the models meeting these criteria, infant size variables were then added to models assessing the effects of infant feeding on hormone levels (Figure 1b: path c'). Results are shown in Table 6.

Among boys, complementary mixed feeding was significantly associated with higher estradiol levels (Table 3, $p=0.01$) and lighter body weight (Table 4, $p=0.01$), which in turn was significantly positively associated with concomitant estradiol levels (Table 5, $p<0.001$ after solids). Adding body weight as a covariate to the regression of feeding on hormone levels (model 1, Table 6) attenuated the association. Thus, controlling for an association between lighter weight and higher estradiol levels reduced the measured effect of feeding on estradiol, although feeding remained marginally significant ($p=0.08$).

Among girls, formula feeding, exclusively or combined with breastfeeding, was at least marginally associated with estradiol levels (Table 3, $p=0.08$, $p<0.001$, respectively) and BMI (Table 4, $p=0.02$, $p=0.03$, respectively). BMI was also significantly negatively associated with concomitant estradiol (Table 5, $p=0.02$). Including BMI in the regression of exclusive formula on estradiol attenuated the association between exclusive formula feeding and estradiol (model 2, Table 6), with feeding remaining marginally significant ($p=0.05$). Including BMI in the model for mixed feeding had no significant effect on the positive association between mixed feeding and estradiol levels (model 3, Table 6; $p=0.001$).

Finally, the more stringent bootstrapping analysis showed no significant indirect effect of any of the size measures on hormone levels (Figure 1b: Path c'). Thus, we found no strong evidence that the relationship between infant feeding and hormone levels was due to a confounding effect of infant size (Table 6).

In summary, we found sex-specific hormonal effects of both fetal and postnatal factors (Figure 2). Among boys, prenatal effects were confined to pregnancy duration and neonatal BMI. Later gestational age at birth was associated with higher estradiol and testosterone levels; greater neonatal BMI accompanied higher testosterone levels during infancy. Among girls, prenatal effects reflected maternal and neonatal size: shorter maternal height and lighter, shorter birth size were associated with higher estradiol levels across infancy. Postnatally, formula use was associated with higher estradiol levels in both boys and girls

compared to those exclusively breastfed before the introduction of solids or to those breast and solid fed after the introduction of solids. Among boys, this effect was present among those exclusively formula fed and those receiving formula in combination with breastmilk and solid foods. Among girls, formula in combination with breastmilk and formula in combination with both breastmilk and solids were associated with higher levels of both estradiol and testosterone levels. In contrast, lower testosterone levels were seen with formula and solid feeding in boys. These associations between infant feeding and hormone levels could not be attributed to an indirect, mediating effect of infant feeding on infant size or body composition.

Discussion

Utilizing novel, non-invasive fecal sex steroid analysis, this study provides the first exploration of energetic correlates of hormonal variation in infancy. These results indicate that pre- and post-natal energetic conditions contribute to variations in estradiol and testosterone levels in infancy, potentially providing a mechanism linking early-life conditions to the tempo of reproductive development and long-term reproductive function.

Specifically, we found a persistent effect of maternal and birth characteristics on fecal sex steroid levels through the first 12 months of life. In addition to these prenatal effects, infant diet was also associated with sex steroid levels, independently of the association between infant feeding and size. These results indicate that both pre- and post-natal energetic conditions contribute to variations in estradiol and testosterone levels in infancy, potentially providing a mechanism linking early-life conditions to the tempo of reproductive development and long-term reproductive function. Further, our results suggest that these energetic environments may differentially affect boys and girls.

Effect of prenatal environment on sex steroid levels

More favorable *in utero* conditions were associated with lower estradiol in girls, but higher testosterone levels in boys across the first year of life. Girls with taller mothers, higher birthweight, or longer birth length, markers of better fetal conditions (Adair and Popkin 1988; Barker and Clark 1997), had lower estradiol levels. Conversely, girls born to first-time mothers, a marker of less favorable uterine environments even in developed countries across a wide range of maternal ages (Ong and Dunger 2002), had higher estradiol levels. This inverse association between estradiol levels and both birth weight and birth length has also been documented in girls (Ruder et al. 2011; Tam et al. 2006) and young women (Finstad et al. 2009) and has been proposed to underlie the association between low birth weight and earlier age at menarche (Ruder et al. 2011; Sloboda et al. 2007). The present report provides the first evidence that these life history trajectories are set prenatally with hormonal effects that are evident in the first year of life.

Among boys, we found a novel positive association between BMI at birth and testosterone levels across the first year of life. No relationship between birth weight and testosterone in the first year was found. This contrasts with previous reports of positive relationships between birth weight and testosterone at later ages (Vanbillemont et al. 2010). Since BMI at birth in boys is associated with greater fat free mass (Butte et al. 2000a), higher BMI may

indicate that good fetal nutritional environments permit both the development of more lean body mass and higher testosterone production. In contrast to a previous retrospective study finding an effect of early postnatal growth, but not birth size, on testosterone levels among adult men (Kuzawa et al. 2010), the present results suggest that the fetal period is also fundamental in organizing the HPG axis among boys, with mechanisms that may include *in utero* exposures and tissue differentiation.

Although the mechanisms linking *in utero* environments to postnatal hormonal levels are unknown, maternal nutrition and hormone levels during pregnancy may affect fetal tissue differentiation, gonad formation and establishment of the endocrine system (Sloboda et al. 2011). Animal models show that experimental manipulation of maternal diet in pregnancy influences the development of gametes in female (Rhind et al. 2001) and male lambs (Bielle et al. 2002) and alters reproductive tempo. Dietary restriction shortens the time to maturation in female rats (Sloboda et al. 2009). Epidemiological research into the developmental origins of reproductive cancers suggests that these long-term associations between maternal characteristics, birth size and sex steroids may reflect exposure to relatively high maternal hormones *in utero* (Finstad et al. 2009; Ruder et al. 2011; Tam et al. 2006; Troisi et al. 2003) altering the development of target organs and gene-signaling pathways (Hilakivi-Clarke and de Assis 2006). While we did not measure maternal hormone levels during pregnancy, our findings are consistent with previous research identifying an inverse association between maternal height and pregnancy estrogens (Wuu et al. 2002), and higher concentrations of estrogens in the umbilical cord blood of first-time mothers (Maccoby et al. 1979; Xue and Michels 2011). Moreover, greater gestational age at birth was associated with higher estradiol and testosterone levels across the first year in boys in our sample. This association was not attenuated when controlling for birth weight, length or BMI, suggesting that these results represent greater exposure to maternal hormones with longer gestation, rather than larger infant size at birth (Troisi et al. 2003).

Effects of feeding type on sex steroid levels

Infant feeding was associated with sex steroids in both male and female infants, albeit in different ways. Breastfed infants had lower estradiol levels than those receiving formula among both boys and girls. The association between formula feeding and testosterone was opposite for boys and girls. Girls receiving formula-- exclusively, with breastmilk, or with breastmilk and solids-- had higher testosterone levels than those not receiving formula. By contrast, complementary formula feeding was associated with lower testosterone in boys. The explanation for these findings remains to be clarified.

To our knowledge, no prior studies have explored an association between infant feeding and concurrent sex steroid production. One previous study documented diet-related differences in reproductive organ size in 4-month old infants, also finding contrasting effects of formula feeding on postnatal reproductive development in girls and boy (Gilchrist et al. 2010). Milk-formula fed girls had greater mean ovarian volumes and a greater number of follicles per cyst per ovary than breast-fed girls, while milk formula-fed boys had lower mean testicular volumes than breastfed boys (Gilchrist et al. 2010). Other studies examining the effects of breastfeeding on long-term reproductive health have found that exposure to breast milk in

infancy is associated with later age at menarche (Ong et al. 2009), lower estrogen and testosterone levels in adulthood (Laustsen et al. 2011), and slightly lower breast cancer risk in adulthood (Borgert et al. 2003; Nichols et al. 2008). While the mechanisms linking breastfeeding to later reproductive outcomes remain to be elucidated, our results suggest that breastfeeding is associated with lower endogenous estrogen production in girls and boys, but higher testosterone production in boys during infancy.

The inverse relationship between breastfeeding and sex steroid levels in our sample and the low concentrations of steroids measured in breastmilk -- 1%–5% of maternal plasma levels (Sahlberg and Axelsson 1986)-- suggest that breastmilk is not the primary source of circulating sex steroids in infants. Rather, formula intake may directly or indirectly augment hormone levels by acting as an exogenous source of hormones, or promoting endogenous steroid production. There is little evidence for a direct effect of exogenous hormones from infant formula (Borgert et al. 2003). Only one infant in this study regularly consumed 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. 1997). Exclusion of this infant did not change study results. Cows' milk formula, consumed by the remainder of formula fed infants, has negligible levels of phytoestrogens (Irvine et al. 1998; Setchell et al. 1997) and the presence and bioavailability of bovine sex steroids in infant formula appears minimal (Irvine et al. 1998; Qin et al. 2007). Thus, an indirect effect of macronutrient differences in breastmilk, formula and complementary foods on hormone production is more likely.

Exclusive breastfeeding is associated with lower energy, protein, and carbohydrate intake at 3 and 6 months compared to formula or mixed feeding (Butte et al. 2000b). Differences in weaning diets may further contribute to differences in macronutrient intakes (Akeson et al. 2000; Koletzko et al. 2009). Energy intakes of formula (Heinig et al. 1993) and mixed fed infants (Haisma et al. 2003) can be as much as 20% higher than that of similarly-aged exclusively breast-fed infants (Haisma et al. 2003). Animal models and epidemiological studies suggest that such energy differences could influence reproductive development. Among male rats, a 20% decrease in dietary energy at weaning inhibited reproductive development and reduced serum testosterone levels (Compagnucci et al. 2002). Greater caloric intake has similarly been associated with higher circulating estrogen levels in adolescent girls and adult women (Dorgan et al. 2003), but not adolescent boys (Dorgan et al. 2006). We did not directly assess energy or macronutrient intakes, and the effects of higher intakes on sex steroid levels in infants have not been previously studied. Two lines of evidence from the present study provide support for an association between infant diet and hormone production: the highest estradiol levels occurred among infants receiving breastmilk, formula and solids, and the highest testosterone levels occurred among girls receiving all three food types.

Infant feeding and size

We hypothesized that formula feeding would be a marker of higher postnatal energy intake based on evidence that formula and solid fed infants have higher energy intake than breastfed infants (Butte et al. 2000b; Haisma et al. 2003; Koletzko et al. 2009). Yet, similar

to a number of studies from developed countries (Butte et al. 2000b; Dewey 2009), we found that formula fed infants had significantly lower BMI and sum of skinfolds than exclusively formula- or mixed-fed infants in the first 4 to 6 months of life. Our findings of larger size in complementary mixed-fed girls also agree with the literature documenting greater weight gain in formula-fed infants in the second half of infancy (Butte et al. 2000b; Dewey 2009). However, our finding that breastfed boys remain larger than complementary formula or mixed fed boys after the first six months diverges from these documented growth patterns. These differences may reflect the high prevalence of on-demand breastfeeding in our sample or the higher energy content of breast milk produced by the mothers of boys (Powe et al 2010). Overall, the discordance between the higher energy intake from formula-feeding vs. breastfeeding, and the larger size of breast fed infants may reflect the influence of parental feeding decisions. Parents alter their feeding practices in response to their perceptions of infant growth (Kramer et al. 2011), introducing reverse causality between size and feeding. Nonetheless, this lack of concordance between feeding type and size in early infancy, as would be predicted if formula was providing greater energy intake, suggests that a more refined marker of energy intake and/or utilization is important for future research.

Differences in infant size do not mediate the association between feeding and hormone levels

In addition to its nutritive content, breast milk provides infants with a host of bioactive factors that may directly influence skeletal growth and body composition. Our results suggest that, although infant feeding is associated with differences in infant size, the effects of infant feeding on sex steroid levels are mostly independent of these size differences. We found only marginal evidence of mediation by infant size in one of the three tested models, and, in each, more conservative bootstrapping analysis did not support a significant indirect pathway through infant size. Instead, infant feeding and size had independent, significant effects on hormone levels. It is possible that feeding and infant size, and body composition in particular, may influence sex steroids through different mechanisms (Schneider et al. 2002). Formula-feeding is associated with differences in plasma insulin (Lonnerdal and Havel 2000), insulin-like growth factor 1 (Chellakooty et al. 2006; Larnkjaer et al. 2009), and leptin (Petridou et al. 2005; Savino et al. 2009) levels which may influence sex steroid production (Rogol 2010). Moreover, feeding differences may contribute to differences in serum cholesterol and lipids (Akeson et al. 2000; Ronis et al. 2011). Formula-fed infants, for example, have increased cholesterogenesis compared to breastfed infants (Bayley et al. 1998; Demmers et al. 2005), with direct implications for sex steroid production (Rogol 2010). Lower levels of cholesterol in the blood inhibit the utilization of cholesterol by the acute steroidogenic regulatory protein (STAR) for steroidogenesis, resulting in decreased serum estradiol and testosterone production (Wang et al. 2005).

Strengths and limitations

The present study is unique in its use of a novel, noninvasive measure of fecal sex steroids, which permitted frequent, repeated hormonal assessment throughout infancy. These longitudinal measures allowed us to examine the concurrent associations between infant feeding, growth and sex steroids on a weekly basis. This sampling frequency provided fine-

grained measures of infant size and sex steroids during the first year of life. Further, our use of weekly 24-hr food diaries likely reduced misclassification of infant feeding type based on recall. Along with these strengths, the present study has a number of limitations. The study had a mixed longitudinal design, which may introduce recall bias in maternal reports of her pre-pregnancy size and infant birth measures due to the differing lengths of time between the index pregnancy and study enrollment. However, maternal recall of birth weight and pregnancy characteristics such as weight gain are highly correlated with medical records even 30–35 years after pregnancy (Tomeo et al. 1999) and improve to nearly 100% at the higher levels of education characterizing our sample (Rice et al. 2007).

The majority of participating infants were breastfed and the small number of observations of exclusive formula feeding (n=17; 3.7% of all feeding observations) and complementary formula feeding (n=54; 11.8% of all feeding observations) may have limited our ability to detect differences between exclusively breastfed and formula fed infants. Although we collected weekly 24-hour diet histories, we did not directly measure infant energy or macronutrient intake; thus, we are unable to determine 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 other macronutrients. Nonetheless, previous research establishing the higher energy intake of formula and solid-fed infants (Butte et al. 2000b; Haisma et al. 2003; Koletzko et al. 2009) and the consistency of the associations between feeding type and hormone levels, suggest that an effect of energy and/or other nutrients on hormone levels is likely. Finally, we cannot definitively conclude that the measured sex steroid levels reflect gonadal, as opposed to extra-gonadal, production since we did not test gonadotropin levels. However, the results of our mediation analysis suggest that these extra-gonadal sources, if present, are not the predominant ones. Greater adiposity, measured through skinfolds or BMI, was not significantly associated with higher estradiol levels in female infants, as would be expected if estradiol was derived from adipose tissue production. Further, infant adiposity was negatively associated with estradiol in male infants, in whom peripheral conversion would account for a greater proportion of total estradiol levels.

Implications for the developmental origins of reproductive function and health

The results of the present study suggest that energy availability *in utero* and that provided through the postnatal diet has an important role in shaping sex steroid levels during early development. In our study, markers of a good fetal environment were associated with lower estradiol levels in girls and poorer uterine environments with higher estradiol levels. This is consistent with implied hormonal correlates of previous research reporting more rapid maturation following poorer uterine environments among girls. In contrast, good uterine environments among boys were associated with higher infant testosterone levels. This sex-specific paradoxical finding may reflect previously documented sex differences in the response of the HPG axis to energetic availability. Among infant boys, like adult men, greater energy availability may permit greater somatic investment in lean body mass (Bribiescas 2001) rather than, or in addition to, accelerated development. Thus, a more favorable uterine environment may provide infant boys with the energy to build more lean body mass. This hypothesis is consistent with our finding that BMI at birth was positively

associated with higher testosterone levels in infancy. These observations have potential functional and evolutionary consequences for later growth and reproductive fitness.

Postnatal feeding patterns also influenced infant hormone levels. Both formula and solid feeding were associated with altered sex steroid levels. Greater estradiol and testosterone were seen in girls and greater estradiol in boys receiving formula before and after the introduction of solid foods compared to breastfed girls and boys, respectively. The linkage between postnatal energy signals and sex steroid production in infancy are a pathway for the establishment of growth and maturational trajectories (Cooper et al. 1996; Ellison 1996) in concert with, or in contrast to, those experienced *in utero*. Our results support the previously documented accelerating effect of good postnatal conditions on reproductive developmental timing.

The sex differences in response to both prenatal conditions and postnatal feeding practices are not unexpected in light of prior research findings. The greater sensitivity of the female HPG axis to energy intake (Ellison 2003) and dietary composition (Dorgan et al. 2003) is well-documented in adolescents and adults (Bribiescas 2001; Ellison 2003; Ellison et al. 1996). The present results suggest that this response may already be present at birth. By contrast, the novel finding of male hormone levels associated with markers of energy availability suggests that early environments may also play an important role in shaping the postnatal testosterone surge, with downstream effects for male reproductive function. Since men experience lower reproductive costs (Bribiescas 2001; Campbell and Leslie 1995), this early energy availability may be an important signal for, or even potential mechanism permitting, lean body tissue accrual. Finally, the lower testosterone levels seen in formula fed boys merit further attention. This finding may highlight the important role of endocrine signals in breastmilk in shaping infant growth and development, the greater energy intake received through breastmilk in boys than girls (Powe et al. 2010), or parental responses of turning to formula for smaller, breastfed male infants.

Taken together, our results provide a potential mechanism linking early life exposures to the developmental origins of reproductive function and health. Energy availability in early life may shape not only early hormone levels, but induce functional and structural changes to the HPG axis with lifelong consequences for both the tempo of development as well as long term sex steroid exposure. With the high levels of formula feeding and early complementary feeding seen in many societies, early excess energy may be an important risk factor for both earlier maturation (Ong et al. 2009) as well as increasing risk for the development of reproductive cancers (Borgert et al. 2003; Nichols et al. 2008). That this hormonal response to energy availability may occur even in conditions of adequate dietary intake across a normal, healthy range of infant weights and body compositions suggests that reproductive development may be sensitive to even subtle fluctuations in energy availability in pre- and postnatal environments. Comparative work in other populations of infants may be critical for understanding the range of variability in infant sex steroid levels expressed in response to energetic environments.

Conclusion

Sex steroid levels across the first year of life reflect maternal prenatal influences and birth characteristics as well as infant feeding practices. This study identifies both fetal and postnatal energetic conditions as influential to early hormone production. Further prospective description of HPG development during infancy is needed. A better understanding of how environmental and social conditions can affect developmental pathways may both provide insights into the key determinants of individual and population level reproductive function and clarify potential consequences of early life environments for the tempo of reproductive development and subsequent lifetime exposure to sex steroids.

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Figure 1.
Analytic strategy for assessing the direct and overall effects of feeding practices on sex steroid levels in infancy

Figure 2.

Association between pre- and postnatal markers of energy availability and sex steroids across the first year of life.

A plus sign indicates a positive association between the variables while a minus sign indicates an inverse association. EFF- exclusive formula feeding, MF-mixed feeding, CFF- complementary formula feeding; and CMF- complementary mixed feeding.

Table 1

Sample Characteristics

	Boys (<i>n</i> =15)		Girls (<i>n</i> =16)	
	Mean (SD)	%(N)	Mean (SD)	%(N)
Maternal Characteristics				
Age (years)	33.4 (4.0)	--	33.4 (4.0)	--
Primiparity	--	60 (9)	--	87.5 (14) ^a
Education (college)	--	92.9 (14)	--	87.5 (14)
Maternal height (cm)	164.8 (6.5)	--	167.2 (7.2)	--
Pre-pregnancy BMI (kg/m ²)	23.1 (2.5)	--	22.9 (2.2)	--
Pregnancy weight gain (kg)	16.4 (5.8)	--	15.8 (6.5)	--
Infant Characteristics				
Gestational age (weeks)	39.2 (1.3)	--	40.1 (1.4) ^a	--
Birthweight (kg)	3.58 (0.37)	--	3.33 (0.45)	--
Birth length (cm)	53.1 (2.2)	--	50.8 (2.7) ^b	--
BMI at birth (kg/m ²)	12.6 (1.2)	--	12.9 (1.5)	--
Infant feeding				
Any breastmilk	--	76.5 (150) ^c	--	89.7 (236) ^c
Any formula	--	68.6 (125) ^c	--	37.6 (99) ^{c,d}
Breastfeeding duration (months)	8.04 (3.3)	--	8.67 (3.6)	--
Age at solid introduction (months)	5.14 (0.62)	--	5.15 (0.97)	--

^a p<0.10 for sex difference from χ^2 tests for dichotomous variables or t-tests for continuous variables

^b p<0.05 for sex difference from χ^2 tests for dichotomous variables or t-tests for continuous variables

^c Frequency represents proportion of weekly feeding measures; number of feeding observations is in parenthesis.

^d Sex differences significant in longitudinal logistic model controlling for repeated measures across subject (p=0.001)

Table 2Association Between Maternal and Birth Characteristics and Infant Sex Steroid Levels Across the First Year ^a

	Estradiol		Testosterone	
	<i>Boys</i> $\beta(p)$	<i>Girls</i> $\beta(p)$	<i>Boys</i> $\beta(p)$	<i>Girls</i> $\beta(p)$
Maternal Characteristics				
Age	-0.04 (0.21)	-0.01 (0.78)	-0.001 (0.97)	-0.02 (0.65)
Maternal pre-pregnancy BMI	0.01 (0.77)	0.05 (0.42)	-0.01 (0.81)	0.03 (0.59)
Pregnancy weight gain	-0.02 (0.34)	0.01 (0.80)	-0.02 (0.53)	0.01 (0.50)
Maternal height	-0.02 (0.22)	-0.03 (0.05)	-0.01 (0.83)	-0.02 (0.16)
Primiparity	0.20 (0.27)	0.73 (0.03)	0.25 (0.38)	0.35 (0.25)
Birth Characteristics				
Gestational age	0.20 (0.00)	-0.06 (0.53)	0.22 (0.03)	-0.04 (0.61)
Birth weight	0.03 (0.92)	-0.51 (0.04)	0.26 (0.51)	-0.34 (0.12)
Birth length	-0.05 (0.28)	-0.10 (0.02)	-0.07 (0.30)	-0.05 (0.17)
BMI at birth	0.09 (0.27)	-0.03 (0.75)	0.24 (0.03)	-0.04 (0.56)

^a Results from longitudinal mixed models with hormone level as the outcome, predicted by maternal and birth characteristics, controlling for age and subject.

Table 3

Association between infant feeding type and hormone levels^a

Feeding Type ^b	Estradiol				Testosterone			
	Boys		Girls		Boys		Girls	
	obs	β(p)	obs	β(p)	obs	β(p)	obs	β(p)
<i>Prior to solids</i>								
EBF	52	ref	66	ref	53	ref	66	ref
EFF	9	0.88 (.002)	8	0.56 (0.08)	9	0.42 (0.49)	7	0.92 (0.09)
MF	17	-0.10 (0.61)	22	0.70 (<.001)	16	0.69 (0.37)	22	1.27 (0.03)
<i>After solids</i>								
CF	37	ref	113	ref	37	ref	112	ref
CFF	36	-1.80 (0.13) ^c	18	0.04 (0.91)	36	-3.41 (0.01)	18	0.94 (0.20)
CMF	43	1.89 (0.01) ^c	36	0.81 (0.003)	43	-1.23 (0.16)	36	1.09 (0.05)

^aResults from longitudinal mixed models with hormone level as the outcome, predicted by infant feeding category, controlling for infant age and repeated measures across subjects.

^bEBF-exclusive breastfeeding, EFF- exclusive formula feeding, MF-mixed feeding, CF- complementary formula feeding, CFF- complementary formula feeding; CMF- complementary mixed feeding

^cModels additionally control for the interaction between infant feeding and age.

Table 4

Association between infant feeding type and size^a

Feeding Type ^b	Size Outcome							
	Length		Weight		BMI		SumSF	
	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
	$\beta(p)$		$\beta(p)$		$\beta(p)$		$\beta(p)$	
EBF	ref	ref	ref	ref	ref	ref	ref	ref
EFF	-2.30 (0.37)	-2.24 (0.02)	-0.16 (0.52)	-0.22 (0.43)	0.01 (0.99)	-1.57 (0.02)	ref	-49.51 (.001)
MF	-0.29 (0.84)	-1.98 (0.13)	-0.58 (0.08)	-0.36 (0.34)	0.04 (0.96)	-1.96 (0.03)	-18.28 (.007)	-22.32 (0.01)
CMF	ref	ref	ref	ref	ref	ref	ref	ref
CFF	1.44 (0.44)	1.44 (0.54)	-0.12 (0.73)	-0.03 (0.97)	-1.10 (0.31)	0.64 (0.23)	-8.42 (.004)	3.46 (0.62)
CMF	-2.74 (0.04)	2.72 (0.03)	-0.65 (0.01)	1.49 (.000)	-1.14 (0.13)	-.0002 (1.00)	-6.32 (0.01)	-3.27 (0.26)

^aResults from longitudinal mixed models with infant size as the outcome, predicted by infant feeding category, controlling for infant age and repeated measures across subjects

^bEBF-exclusive breastfeeding, EFF- exclusive formula feeding, MF-mixed feeding, CMF- complementary formula feeding, CFF- complementary mixed feeding; SumSF- sum of skinfolds.

^cModel could not converge due to the small number of EFF infants with sumSF measures

Table 5Association between infant size and hormone levels^a

	Hormonal Outcome			
	Estradiol		Testosterone	
	Prior to solids			
	Boys	Girls	Boys	Girls
	β(p)	β(p)	β(p)	β(p)
Length	−0.13 (0.01)	−0.05 (0.27)	−0.0003 (1.00)	−0.06 (0.31)
Weight	−0.83 (0.02)	−0.16 (0.26)	−0.29 (0.26)	0.12 (0.52)
BMI	−0.36 (0.07)	−0.19 (0.02)	0.25 (0.21)	−0.04 (0.67)
Sumsf	−0.03 (0.16)	0.02 (0.31)	0.05 (0.05)	−0.01 (0.67)
	After solids			
	Boys	Girls	Boys	Girls
	β(p)	β(p)	β(p)	β(p)
Length	−0.09 (0.46)	0.07 (0.34)	0.02 (0.86)	−0.06 (0.33)
Weight	−0.91(0.00)	−0.05 (0.85)	−0.56 (0.03)	−0.23 (0.20)
BMI	−0.93 (0.00)	−0.03 (0.87)	−0.51 (0.01)	−0.15 (0.31)
Sumsf	−0.09 (0.07)	0.03 (0.47)	0.02 (0.72)	0.01 (0.78)

^aResults from longitudinal mixed models with hormone level as the outcome, predicted by infant size, controlling for infant age and repeated measures across subjects

Table 6

Mediation analysis of the effect of infant size on the association between infant feeding and hormone levels^a

Model	Predictor (p)	Mediator (m)	Outcome	$\beta_p(p)^b$	$\beta_m(p)^c$	Bootstrapping indirect effect ^d
<i>Boys</i>						
1	CMF ^e	--	EST	1.86 (0.01)	--	N
	CMF	Weight	EST	1.45 (0.08)	-0.49 (0.00)	
<i>Girls</i>						
2	EFF	--	EST	0.56 (0.06)	--	N
	EFF	BMI	EST	0.62 (0.05)	0.03 (0.62)	
3	MF	--	EST	0.75 (0.00)	--	N
	MF	BMI	EST	0.82 (0.001)	0.08 (0.29)	

^a Results from mixed models testing the direct and indirect effects of infant feeding type on hormonal outcome, controlling for infant age and repeated measures across subjects

^b Represents the coefficient and p value of the predictor variable

^c Represents the coefficient and p value of the mediator variable

^d Evidence for a significant indirect effect from bootstrapping models (Krull and MacKinnon 2001)

^e EFF- exclusive formula feeding, MF-mixed feeding, CMF- complementary mixed feeding, EST- estradiol