Maternal Gestational Androgen Levels in Female Marmosets (*Callithrix geoffroyi*) Vary Across Trimesters but Do Not Vary With the Sex Ratio of Litters

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Abstract

Maternal hormones can dramatically modify offspring phenotypes via organizational actions on morphological and behavioral development. In placental mammals, there is the possibility that some portion of hormones in maternal circulation may be derived from fetal origin. We tested the possibility that maternal androgens in pregnant female marmosets reflected, in part, contributions from male fetuses by comparing levels of urinary androgens across pregnancy in females carrying varying numbers of male offspring. We monitored urinary androgen excretion in 18 pregnancies from five female white-faced marmosets (*Callithrix geoffroyi*). Androgen levels rose significantly in the first trimester of pregnancy, reached a peak in the middle of the second trimester, and then declined gradually until parturition. At no point in pregnancy were levels of urinary androgens higher in females carrying litters that had 50% or more males than females carrying litters that were less than 50% male. Levels of maternal androgens were not associated with litter size, the number of males in the litter, or with the proportion of the litter that was male. The high levels of androgen in pregnant females are therefore likely of strictly maternal origin, and any modification of fetal growth and development can be considered a 'maternal effect'.

1. Introduction

Steroid hormone production during pregnancy in female mammals is typified by dramatic increases in estrogens and progestagens, derived from ovarian, luteal, and placental sources (Albrecht & Pepe, 1990; Ojeda, 2004). The onset of pregnancy in a number of species is also associated with concomitant increases in the production of androgens (dog: Concannon & Castracane, 1985; baboon: Castracane & Goldzieher, 1983; human females: Castracane & Asch, 1995; Castracane et al., 1998; rhesus macaques: Challis et al., 1975; marmoset: Chambers & Hearn, 1978; Fite et al., 2005; rat: Gibori & Sridaran, 1981; Legrand et al., 1984). There is a growing interest in the possibility that maternally-derived androgens can significantly impact embryonic and fetal development in vertebrates (Groothuis et al., 2005; Dloniak et al., 2006; Hines, 2006). Developing male fetuses also produce androgens (Parker,

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2004) that may move from the fetal compartment to the maternal compartment via placental vasculature. In order to adequately assess strictly 'maternal effects' of gestational androgens, therefore, it is critical to determine whether the measurement of androgens from maternal biological samples reflect steroids of solely maternal origin, or of maternal plus fetal origin.

There is some controversy whether androgens of fetal origin enter maternal circulation in concentrations sufficient to alter maternal levels. There are a number of studies that show that maternal androgens are higher in human mothers when she is carrying a male fetus than when she is carrying a female fetus (Muelenberg & Hofman, 1992; Nagamani et al., 1979), with significant differences emerging as early as five to seven weeks post-conception (Klinga et al., 1978; Harrison & Mansfield, 1980). Similar differences in maternal androgens have also been reported in baboons (Altman et al., 2004), elephants (Duer et al., 2002), and lemurs (Ostner et al., 2003). However, there is also evidence that maternal androgen concentrations are not affected by the sex of the fetus the mother is carrying (Glass & Klein, 1981; Steler et al., 2002; Triosi et al., 2003). Several recent studies have also demonstrated no effect of fetal sex on circulating maternal androgens. Two studies contrasted androgen concentrations in amniotic fluid or cord blood, as well as in general maternal circulation, in human mothers carrying males or females. As expected, androgens were higher in both amniotic fluid and cord blood for male fetuses than female fetuses, but there was no evidence that fetally-derived androgens altered maternal endocrine states, since circulating androgens did not differ as a function of fetal sex (Triosi et al., 2003; Van de Beek et al., 2004). Finally, Cohen-Bendahan et al. (2005) evaluated maternal testosterone levels in women carrying same-sex or opposite-sex twins, and found that maternal androgens did not vary significantly as a function of the sex composition of the twins. In a litter-bearing rodent (rat), levels of maternal androgen do not vary systematically with the sex ratio of the litter (Castracane & dela Cruz, 1990; Houtsmuller et al., 1995). In rats, normal variation in litter size is not associated with variation in circulating maternal androgens (Houtsmuller et al., 1995), but experimental reduction of litters early in gestation to four or one implanted embryos is associated with a significant reduction in maternal androgen (Castracane & dela Cruz, 1990).

Marmosets of the genus *Callithrix* provide a useful model for assessing the potential influence of maternal physiology on developing offspring. First, marmosets are the only simian primates that produces a litter. The modal litter size is two, but litter sizes can range from one to five (Smucny et al., 2004; Ross et al., 2007). Second, since the offspring are not identical twins, the sex ratios of litters can vary from 0% to 100% males. Finally, it has already been demonstrated that marmoset females, like other primates, exhibit elevated levels of circulating androgens during gestation (both testosterone (T) and androstenedione (A_4)), with peak concentrations occurring during the second trimester (Chambers & Hearn, 1979). However, it is important to determine whether measures of maternal androgen concentrations during gestation reflect solely maternal sources, via ovarian or placental steroidogenesis, or reflect both maternal and fetal contributions.

In the present study, we evaluated androgen profiles in female marmosets throughout pregnancy, and the impact of litter sex ratio on levels of excreted urinary androgens in female marmosets. Urine was collected from female marmosets throughout pregnancy, and we examined the resulting endocrine profiles as a function of the sex ratio of the litter carried by the mother. To the extent that male fetuses constitute a source of androgen for their mothers, we predicted that mothers with male fetuses, or a greater proportion of fetuses that were male, would have higher levels of excreted urinary androgens. We also evaluated the impact of overall litter size (regardless of sex ratio) and maternal age on androgen levels.

2. Methods

2.1 Subjects

Five female marmosets (*Callithrix geoffroyi*) provided samples for this study. The marmosets were housed in the Callitrichid Research Center at the University of Nebraska at Omaha. All females were housed with an adult male pairmate, and from zero to eight offspring. Table 1 provides demographic information on the females in the study, including female ages and the sex ratios of their litters. Pairs and family groups were housed in large enclosures (minimum: $0.6 \times 0.6 \times 2.2$ m; maximum: $3.0 \times 3.0 \times 2.5$ m) that varied depending upon groups size, and contained natural branches, nest boxes, and feeding stations. Rooms were maintained on a 12h: 12h light:dark cycle, with light onset occurring between 0700 and 0800 h. Marmosets had *ad lib* access to water, and were fed a varied diet of commercial marmoset chow, fresh fruits and vegetables, and animal protein once each day. The routine research and husbandry procedures in our facility were designed to minimize disruptions and disturbances to the day-to-day activities of the marmosets. Details of colony management and husbandry can be found in Schaffner et al., (1995). All of the procedures were evaluated and approved by the University of Nebraska at Omaha/University of Nebraska Medical Center Institutional Animal Care and Use Committee: Protocols 01-022-03 and 07-033-05.

2.2 Selection of pregnancies

We used the following criteria to identify candidate pregnancies for this study. First, we selected only those pregnancies that went to full-term, either on the basis of the estimated length of gestation as determined from hormone profiles or on the presence of full-term infants (perinatal weights > 30 g). Second, we eliminated any pregnancies that were known or suspected to have had early fetal loss. Finally, we eliminated any pregnancies in which the sex of all offspring in the litter could not be unambiguously determined. The application of these criteria yielded 18 pregnancies from the five females.

2.3 Urine collection

We collected urine samples from the breeding females two to five times per week continuously throughout the study period. A non-invasive, stress-free procedure was used as described previously (French et al., 1996). Briefly, animals were trained, using reward conditioning, to provide a urine sample. Samples were collected in disposable aluminum pans, immediately after light onset in the colony rooms, and hence represented first-void samples. After collection, the samples were centrifuged at 2000 rpm for two minutes to separate solid detritus, and the clean supernatant was then transferred to a clean 1.5 ml minivial for storage. All samples were cataloged in our database and kept frozen at $-20°$ C until assayed.

2.4 Androgen assay

Concentrations of maternal androgen were determined by assaying urine samples via a testosterone enzyme immunoassay that has previously been characterized and validated for marmosets (Nunes et al., 2000; Fite et al., 2005). Briefly, urine samples (10 µl) were extracted in 5 ml freshly-opened diethyl ether after enzyme hydrolysis with β-glucuronidase (Sigma Chemical, St. Louis MO). The ether was evaporated in a warm water bath under a gentle stream of air, and samples were reconstituted in 1.0 ml phosphate buffered saline. Procedural losses of androgen during the extraction procedure were monitored by the recovery of radiolabelled testosterone, and sample concentrations were corrected for loss. Each microtiter plate contained a standard curve (1000 – 7.8 pg testosterone), high and low concentration quality controls consisting of pooled marmoset urine, and samples. All standards, quality controls, and samples were assayed in duplicate. The testosterone antibody used in the assay system cross-reacts with androstenedione and dihydrotestosterone (Dloniak et al., 2004), hence the results are described

as urinary androgen concentrations, rather than urinary testosterone. We used two sets of quality control pools, and interassay coefficients of variation averaged 17.9% and 7.4% for the high and low concentration pools, respectively. Intra-assay coefficients of variation for the same pools averaged 7.9% and 10.4% for the high and low concentration pools, respectively. In order to control for variable fluid intake and urinary output, all androgen concentrations were corrected by urinary creatinine concentrations. We used a modified Jaffé end-point assay (Tietz, 1976) which has been previously described and validated for marmosets (French et al., 1996).

2.5 Statistical analyses

We conducted several analyses to assess whether the sex ratio of the litter was associated with levels of excreted androgens in pregnant female marmosets. First, we divided pregnancies into those in which 50% or more of the litter were male fetuses (n = 12) and those in which less than 50% of the litter were male fetuses (n = 6). For each pregnancy, we calculated pre-pregnancy levels of androgen excretion immediately prior to conception, and for 10-day blocks during the 146 – 149 day gestation period. The values for each 10-day block represented the androgen levels averaged from one to five samples collected during each block. A mixed-model ANOVA was used to assess differences across gestation as a function of the sex ratio of the litter. "Female" was included as an independent effect, and multiple pregnancies from the same female were nested under the Female effect. In this way, we could also assess individual differences among females in androgen excretion profiles throughout pregnancy. We also conducted a similar mixed-model ANOVA across 10-day blocks of gestation but contrasted excreted androgens in litters that only contained males (n = 4) versus litters that only contained females (n = 3).

Second, we conducted correlational analyses to assess whether continuous variation in litter sex ratio was predictive of maternal androgen excretion. Toward this end, we calculated nonpregnant androgen levels for each pregnancy and a mean concentration for each trimester of pregnancy. Means for the trimesters derived from androgen concentrations from more than 10 – 20 samples that were distributed evenly throughout each trimester. These values were then correlated with two estimates of sex ratio: (1) the number of males in the litter (range: 0 – 4) and the proportion of the litter that was male (range: 0 – 1.0). To the correlational analyses we also added female age and litter size, to see if any variation in androgen concentrations could be explained by these variables.

3. Results

Gestational status was associated with significant variation in excreted maternal androgens (Fig. 1). Concentrations were low prior to conception (Day 0), then rose early in pregnancy, reaching maximal concentrations during the second trimester (days 50 – 100). Excreted androgens dropped rapidly during the third trimester, returning to near pre-pregnancy levels in the final 10 days of gestation. The differences in androgen excretion across gestation were highly significant ($F(15,150) = 13.93$, $p < 0.001$), and post-hoc comparisons revealed that relative to preconception levels, excreted androgens were significantly higher at all times during gestation, including the period just prior to parturition (p's < 0.005). Patterns of androgen excretion did not vary as a function of the sex composition of the litter, as the interaction between gestational stage and litter sex composition was not significant ($F(15, 150) = 1.21$, n.s.; see Fig. 1). When contrasts were limited to those pregnancies in which marmoset females were carrying male-only versus female-only litters, an identical pattern was noted. That is, there were significant changes across gestation ($F(14,70) = 3.68$, $p < 0.001$), but androgen levels did not vary at any time point based on litter sex composition. However, females differed from each other in the pattern of androgen excretion throughout gestation (F

$(60,150) = 2.46$, p < 0.001). Some females (e.g., Bes, Swe) showed moderate elevations in androgen during gestation, while other females (e.g., Dar, Pop) exhibited five- to eight-fold increases in androgen excretion, relative to nonpregnant concentrations.

Our correlational analyses also indicated that litter sex ratio has no effect on maternal androgen. As seen in Table 2, neither the number of male fetuses carried by the female, nor the proportion of the litter that was male, was significantly associated with maternal androgen concentrations at any stage of gestation. In fact, androgen levels in the third trimester tended to be lower in females whose litters had a higher proportion of male fetuses ($r = -0.23$, n.s.), but the relationship did not reach conventional levels of significance. Variation in litter size (range: 1 – 4; M $\pm$ sem: 2.24 ± 0.21) was not associated with maternal androgen excretion. Finally, maternal age (and parity, since both variables covary) did not predict maternal androgen excretion during gestation. However, baseline androgen concentrations in nonpregnant females were significantly associated with age ($r = 0.56$, p < 0.02), with older females exhibiting higher concentrations than younger females. Levels of androgen excretion during nonconceptive periods were not associated with subsequent levels during pregnancy, but levels in adjacent trimesters ($1^{st} – 2^{nd}$, $2^{nd} – 3^{rd}$) were significantly related to each other. However, levels in the 1st trimester were only weakly correlated with levels in the 3rd trimester ($r = 0.41$, p < 0.10).

4. Discussion

Maternally-derived hormones have the potential to dramatically organize and modify behavioral and morphological trajectories in developing organisms. This study revealed that pregnant female marmosets have elevated levels of excreted androgens. Androgen levels rose during the first trimester, reached peak concentrations in the second trimester (~ 80 days post-conception), and gradually declined during the third trimester. There were no differences in maternal androgen as a function of the sex composition of the female's litter, as analyzed either by levels associated with the presence or absence of a male fetus, or as a function of the number or proportion of males in the litter. Litter size was not related to variation in gestational androgen concentrations, nor was female age and parity. However, nonconceptive androgen levels were higher in older females than in younger females. Since excreted androgens correlate highly with circulating androgens in marmosets (Nunes et al., 2002), these results suggest that maternally derived androgens could exert a masculinizing influence on offspring *in utero*.

The finding that maternal androgen levels throughout pregnancy did not differ as a function of litter sex ratio is in contrast with early studies on human females (e.g., Muelenberg & Hofman, 1991; Klinga et al., 1978), but is consistent with more recent reports (Van de Beek et al., 2004). Our study parallels the study of human mothers carrying same-sex or opposite-sex twins (Cohen-Bendahan et al., 2005). While levels of maternal testosterone and estradiol rose throughout the mothers' pregnancies, mothers carrying twin males or mixed sex twins did not have higher circulating androgens at 24 or 32 weeks of gestation than mothers carrying twin females. In humans and Old World monkeys, fetal testes begin synthesizing and secreting androgens in the first half of pregnancy (35 – 50 days in macaques: Resko & Ellingwood, 1981; 40 – 60 days in humans: Parker, 2004). There are no published data on the timing of testicular production of androgens in male marmoset fetuses, but some androgen-driven sexual differentiation clearly occurs during fetal development, since neonatal males have differentiated primary sexual characteristics (McKinnell et al., 2001). There may be some postnatal behavioral and morphological plasticity, however, since treating female marmosets with perinatal androgen masculinizes external genitalia and sexual and aggressive behavior (Abbott & Hearn, 1979).

While the present study did not explicitly examine the origin of urinary androgens in pregnant female marmosets, it is likely that they derive from multiple sources, including ovarian and/

or corpora luteal, placental, and adrenal tissue. There are increases in plasma T and A_4 and urinary androgen excretion in pregnant female marmosets within one to two weeks of conception, prior to implantation and the elaboration of the fetoplacental unit (Chambers & Hearn, 1979; Fite et al., 2005). These patterns suggest that early gestational androgen is of ovarian origin. Ovarian steroidogenesis in the immediate post-conceptive period in a number of mammalian species is characterized by elevated circulating maternal testosterone levels (dog: Concannon & Castracane, 1985; baboon: Castracane & Goldzieher, 1983; human females: Castracane & Asch, 1995; Castracane et al., 1998). In the baboon, elevated androgen production by the ovary appears to be under the regulation of chorionic gonadotropin (CG) stimulation, since administering hCG to nonpregnant females late in the luteal phase elevates androgen concentrations to levels that are characteristic of pregnant baboons (Castracane & Goldzieher, 1983). Gonadotropin levels in pregnant marmosets begin to rise 13 – 17 days after conception, coincident with increasing androgen production, and peak in early to mid second trimester, when androgen levels are also peaking (Chambers & Hearn, 1979; French et al., 1996; present study), suggesting that gonadotropins are important for regulating androgen production in marmosets, as well.

Direct evidence for placental production of androgens is not available, but the production of other steroids suggests a shift from gonadal to placental hormone production well before we documented peak levels of urinary androgen in pregnant marmosets. By comparing circulating levels of progesterone and estradiol with levels sampled from the utero-ovarian vein, Hodges et al. (1983) noted that both the corpus luteum and placenta were contributing to circulating progesterone at 40 days post-conception, but that luteal production of progesterone has ceased by day 60. To the extent that androgen production follows a similar pattern, the peak levels of urinary androgens we noted at day 80 of gestation is well past the shift from luteal to placental steroid production.

Molecular evidence for the role of the placenta in androgen steroidogenesis is also provided by the timing and distribution of mRNA for a critical steroid enzyme, 17β-hydroxysteroid dehydrogenase (HSD) in reproductive tissues. This enzyme converts androstenedione (A_4) to T, and estrone (E_1) to estradiol (E_2). mRNA for one variant of the enzyme (HSD1) is found extensively in marmoset placenta, and mRNA for aromatase is co-localised with HSD1 mRNA. A second variant of HSD (HSD7) is widespread in corpora lutea (Husen et al., 2003). Conception status also affects the presence and distribution of mRNA for HSD. HSD7 mRNA is not expressed in the uteri of marmosets in the luteal phase of nonconceptive cycles, but is massively up-regulated in the uterus of female marmosets 13 to 15 days after conception, especially in the uterine endometrium near the implantation sites (Einspanier et al., 2009). The marmoset ovary, uterus, and placenta are active sites for both androgen and estrogen biosynthesis.

We noted in the present study that baseline, nonpregnant levels of androgen excreted by female marmosets increased with a female's age. In many species, aging in females is associated with reduced, not elevated, circulating androgen concentrations. In human females, testosterone and especially androstenedione decline with age (Elmlinger et al., 2005; Davison et al. 2005), but the decline in circulating androgens is gradual, and there is no discontinuity at the time of menopause (Davison et al., 2005). Androgen excretion is also reported to decline in wild female baboons, but only when exceedingly old females are included in the sample (Beehner et al., 2005). Androgens become elevated in older female rats (25 months of age), but declines again when exceedingly old females experience reproductive senescence (Lu et al., 1979; Goya et al., 1990), but this effect is seen only in very old rats (25 – 30 months of age). Female marmosets exhibit signs of follicular depletion and hence a cessation of ovulation at or near the expected lifespan, so they do not exhibit significant age-related declines in reproduction (Tardif et al., 2008). Unlike Old World and hominid primates, however, the steroidogenic capacity of the

aging callitrichine primate (marmosets and tamarins) ovary does not show a decline, since levels of estrogens and progestagens are similar to or higher in older females than in younger females (Tardif et al., 2008; Tardif & Ziegler, 1992). Our results suggest that the maintenance and/or enhancement of steroidogenic capacity in older females holds for androgens as well as other ovarian and placental hormones. That being said, however, the age range of female marmosets in our study was relatively restricted (4.5 – 8.7 years of age). These ages are not exceptionally old for a species in a genus (*Callithrix*) that can live to more than 16 years of age (Ross et al., 2007).

In summary, our results show that excreted urinary androgen concentrations in pregnant female marmosets are not altered by the presence of one or more male fetuses *in utero*. The elevated levels of androgens in pregnant females appear to be of maternal origin only, and are likely to be of ovarian and placental origin. The uterine endocrine environment can have profound impacts on morphological and behavioral phenotypes in mammals, and the role of prenatal androgens in shaping sex-typical traits is well established (Morris et al., 2004; Wallen, 2005). Given that there is considerable variation both within and among females in levels of androgen during gestation, the marmoset represents a potent model in which to examine maternal effects on offspring development that results from prenatal androgen exposure.

Acknowledgments

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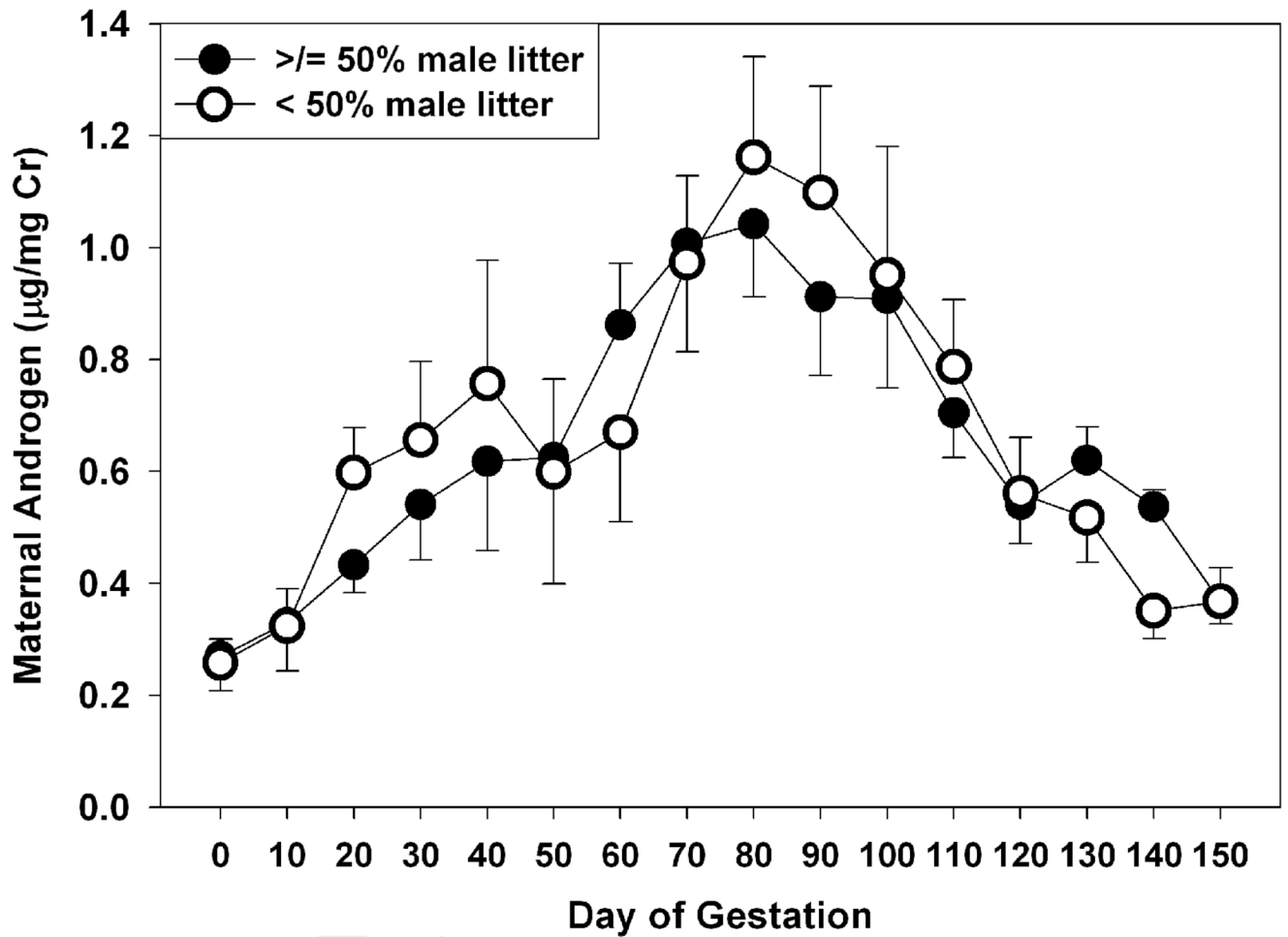
Fig. 1.
Patterns of urinary androgen excretion across gestation in female white-faced marmosets.
Values indicate mean ± s.e.m. Filled circles represent females that were carrying litters that
were 50% or more males (n =12); open circles represent females whose litters were less than
50% male fetuses (n = 6). Day 0 reflects mean androgen excretion in nonpregnant females
immediately prior to conception. See Table 1 for additional details.

Table 1

Subjects and Demographic Characteristics

Female	Age Range (years)	Sex Ratios of Litters in Study[a]
Bes	4.6 – 6.6	1.1, 1.0
Dar	4.8 – 5.7	1.1, 0.2, 1.1
Lor	5.7 – 6.6	1.2, 0.2, 1.0
Pop	4.7 – 8.7	1.1, 4.0, 2.1, 2.0, 2.1
Swe	4.5 – 6.7	1.1, 1.2, 0.2, 1.1, 1.3

[a]Numbers represent males.females in litter

Table 2

Correlations Among Maternal Gestational Androgens and Litter Characteristics

Gestational Phase[a]	Tri1	Tri2	Tri3	# Males	Prop. Male	Lit Size	Age[b]
NonPreg	0.37	0.09	0.17	0.02	0.10	0.02	**0.56**[d]
Tri1	-	**0.70**[c]	0.41	0.28	0.11	0.34	0.30
Tri2	-	-	**0.74**[c]	0.16	−0.08	0.17	0.04
Tri3	-	-	-	−0.11	−0.23	−0.17	0.22

[a] Maternal androgens were averaged for periods in which the female was not pregnant, and in each trimester of pregnancy (Tri1, Tri2, and Tri3)

[b] Maternal age is also a proxy variable for female parity

[c] $p < 0.001$

[d] $p < 0.02$