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Sexual Dimorphism in Titi Monkeys' Digit (2D:4D) Ratio is Associated with Maternal Urinary Sex Hormones During Pregnancy

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Abstract

The second-to-fourth digit (2D:4D) ratio is a sexually-dimorphic biomarker for prenatal sex hormone exposure. We investigated whether titi monkeys (*Plecturocebus cupreus*) exhibit sexually-dimorphic 2D:4D ratio, and whether variation in 2D:4D ratio correlates with maternal testosterone and estrogen levels during early pregnancy. Subjects were 61 adult titi monkeys (32 males, 29 females). For 26 subjects, maternal urine samples were collected approximately 15–20 weeks before birth and assayed for testosterone and estrone conjugate (E_1C). Titi monkeys exhibited a human-like pattern of sexual dimorphism in right-hand 2D:4D ratio, with females exhibiting higher 2D:4D ratio than males ($\beta = -0.29$, $p = .023$). For left-hand 2D:4D ratio, high levels of maternal E_1C predicted low offspring 2D:4D ratio ($\beta = -0.48$, $p = .009$). For right-hand 2D:4D ratio, high levels of testosterone ($\beta = -0.53$, $p = .005$) and testosterone-to-E_1C ratio ($\beta = -0.41$, $p = .028$) predicted low offspring 2D:4D ratio. For 2D:4D ratio asymmetry (right-hand – left-hand), high levels of testosterone ($\beta = -0.43$, p = .03) and testosterone-to-E_1C ratio ($\beta = -0.53$, $p = .003$) predicted low (right-biased) asymmetry. This is the first report of sexually-dimorphic 2D:4D ratio in New World monkeys, and the results support a growing literature suggesting prenatal sex hormones may modulate offspring 2D:4D ratio.

Keywords

2D:4D Ratio; Titi Monkey; Sexual Dimorphism; Prenatal Androgen; Prenatal Estrogen; Maternal Hormones; Urinary Sex Hormones

Introduction

Prenatal androgens and estrogens organize the course of body and brain development, contributing to lasting sex differences in brain structure and behavior (Ehrhardt & Meyer-

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Bahlburg, 1979; MacLusky & Naftolin, 1981). Although prenatal androgens are produced primarily by the testes of the male fetus and are principal hormones that masculinize male genitalia and body morphology (Hughes, 2001), both male and female fetuses experience prenatal androgen exposure from secondary sources. For example, some androgens are produced as byproducts when other steroids are synthesized, and some androgens may originate from the mother (see Knickmeyer & Baron-Cohen, 2006). Although these levels of androgen exposure are typically not high enough to lead to virilization of females' genitalia, they could lead to individual differences within females. Prenatal estrogens are also produced endogenously by both male and female fetuses (Abney, 1999; Beyer, 1999), and have organizational effects on the reproductive organs, particularly in females (Lubahn et al., 1993).

Beyond these morphological effects, prenatal sex hormones affect brain development (Beyer, 1999). Even before birth, androgen and estrogen receptors are widely distributed throughout the developing brain (Vito & Fox, 1981). By acting on these receptors, prenatal sex hormones affect DNA expression, cell growth, axonal and dendritic projections, and neurotransmitter production (see Beyer, 1999; MacLusky & Naftolin, 1981; Morris, Jordan, & Breedlove, 2004). These early organizational effects on the central nervous system may contribute to the development of sexually dimorphic personality traits like aggression (Bailey & Hurd, 2005; Benderlioglu & Nelson, 2004), competitiveness (Mailhos, Buunk, Del Arca, & Tutte, 2016; Pokrywka, Rachoń, Suchecka-Rachoń, & Bitel, 2005), and overconfidence (Dalton & Ghosal, 2018). Extreme levels of exposure may also be linked with neurodevelopmental disorders, including autism spectrum disorder (Manning, Baron-Cohen, Wheelwright, & Sanders, 2001), schizophrenia (Collinson et al., 2010; Paipa et al., 2018), anxiety disorder (Evardone & Alexander, 2009), and attention deficit hyperactivity disorder (Stevenson et al., 2007). A better understanding of how prenatal sex hormones affect neurological and behavioral traits may lead to better treatments and interventions for these disorders.

To study the long-term effects of prenatal sex hormone exposure, the second-to-fourth-finger-length (2D:4D) ratio is commonly used. Human males tend to exhibit a longer ring finger (4D) when compared to the pointer finger (2D), while females tend to exhibit the opposite pattern, with the second digit longer than or equal in length to the fourth digit. Hence, on average, human males tend to exhibit lower 2D:4D ratio than females (see Manning, 2011). The reason for this pattern of sexual dimorphism has traditionally been ascribed to a combination of three factors: (1) males experience higher levels of prenatal androgens than females, (2) prenatal androgens promote finger growth, and (3) prenatally, androgen receptors are more highly concentrated in the fourth digit than in the second digit (Manning, 2011; Zheng & Cohn, 2011). Based on these interacting factors, it is hypothesized that, as androgens masculinize the typical male fetus, they induce greater growth in the fourth digit than in the second digit because the fourth digit has more receptors for androgen binding than the second digit (Zheng & Cohn, 2011). This theory also explains why males, on average, exhibit lower 2D:4D ratio than females. Because the sensitive period when 2D:4D ratio is determined occurs early in gestation during the same period when the brain is most sensitive to prenatal sex hormones (see Manning, 2011), this markers provide a unique window to gestational development. Prenatal estrogens also affect 2D:4D ratio.

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Prenatally, estrogen receptors are, like androgen receptors, more highly concentrated in the fourth digit than in the second digit (Zheng & Cohn, 2011). However, unlike androgens, prenatal estrogens inhibit finger growth (Zheng & Cohn, 2011), and more so for the fourth digit than the second digit, due to the differential receptor expression between the fingers. Consequently, prenatal estrogens are thought to correlate positively with 2D:4D ratio (Manning, 2011; Zheng & Cohn, 2011), with high levels of prenatal estrogens leading to higher (female-typical, in humans) 2D:4D ratio. However, because prenatal estrogens do not, on average, differ between male and female fetuses (Beyer, 1999; Çetin, Can, & Özcan, 2016; Lutchmaya, Baron-Cohen, Raggatt, Knickmeyer, & Manning, 2004; Mitsui et al., 2015; however, see Hollier et al., 2015), 2D:4D ratio may be determined by prenatal androgen-to-estrogen level ratios altering the length of the fourth digit, with sex differences in levels of prenatal androgens driving sexual dimorphism in 2D:4D ratio, and prenatal estrogen levels attenuating it (Lutchmaya et al., 2004; Manning, 2011).

Although this process is likely similar for left-hand 2D:4D ratio and right-hand 2D:4D ratio, the effects of prenatal sex hormones may be stronger for the right hand than for the left. For example, in humans, right-hand 2D:4D ratio tends to exhibit a greater degree of sexual dimorphism than left-hand 2D:4D ratio (Hönekopp & Watson, 2010). Several other studies have shown that in both men and women the right-hand 2D:4D ratio is more consistantly linked with androgen-related phenotypes, including fertility measures (Manning, Scutt, Wilson, & Lewis-Jones, 1998), competitiveness (Bönte, Procher, Urbig, & Voracek, 2017), and aggression (Hampson, Ellis, & Tenk, 2008; however, this association may be moderated by sex, and other evidence is mixed—see Turanovic, Pratt, & Piquero, 2017). These findings are congruent with studies of other body parts showing that, in both women and men, masculine phenotypes may be more apparent on the right side of the body and female phenotypes are more apparent on the left side of the body (see Kimura, 1994; Manning, 2002; Tanner, 1990; Zaidi, 2011), and suggest that prenatal sex hormones contribute to body asymmetry. For this reason, 2D:4D ratio asymmetry (elsewhere referred to as Dr-l), calculated as the right-hand 2D:4D ratio minus the left-hand 2D:4D ratio, is commonly used as an additional measure of prenatal sex hormone exposure, with negative asymmetry (i.e., asymmetry < 0, or right-hand 2D:4D ratio < left-hand 2D:4D ratio) suggesting high levels of prenatal androgen exposure (Manning, 2002). This interpretation is supported by studies showing that low 2D:4D ratio asymmetry is associated with masculinized phenotypes, including high levels of indirect aggression in women (Coyne, Manning, Ringer, & Bailey, 2007) and greater athletic ability in men (Voracek, Reimer, Ertl, & Dressler, 2006).

The traditional explanation for sexually dimorphic 2D:4D ratio in humans described above is supported by experimental research in rodents (Zheng & Cohn, 2011) and was recently expanded to explain why some species exhibit a reversed pattern of 2D:4D ratio compared to humans (see Baxter et al., 2018; Lofeu, Brandt, & Kohlsdorf, 2017). Although relatively few studies have investigated 2D:4D ratio in nonhuman primates, studies thus far suggest that 2D:4D ratio varies across primate families (Nelson & Shultz, 2010), with some Old World (OW) species exhibiting, on average, lower 2D:4D ratio than humans (McIntyre et al., 2009; Nelson & Shultz, 2010). The direction of sexually dimorphic 2D:4D ratio also varies within each species. Humans' closest relatives, chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*), exhibit a human-like pattern of sex differences in 2D:4D ratio (males exhibit

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lower 2D:4D ratio than females; see McIntyre et al., 2009), while some Old World species like rhesus monkeys (*Macaca mulatta*) (Abbott, Colman, Tiefenthaler, Dumesic, & Abbott, 2012; Baxter et al., 2018) and baboons (*Papio papio*) (Roney et al., 2004) show a reversal from the human pattern of sexual dimorphism in 2D:4D ratio (males exhibit higher 2D:4D ratio than females). Some propose that the reason for this reversed pattern is that OW monkeys may have a reversed expression rate of sex hormone receptors in the fingers, with greater receptor density in the second digit rather than in the fourth digit (see Baxter et al., 2018; Lofeu et al., 2017). If this is true, the magnitude and direction of 2D:4D ratio sexual dimorphism likely depends on species-specific patterns of sex hormone receptor density in the fingers (Lofeu et al., 2017). Lofeu and colleagues (2017) theorized that, as species evolved, random mutations determined which finger had greater sex hormone receptor density and, during evolution, these mutations were pleiotropically selected for along with other sexually-dimorphic traits. Considering that studies of 2D:4D ratio and direct measures of exogenous prenatal hormones have been conducted almost exclusively in humans and have yielded mixed results (for review, see Manning & Fink, 2017; Richards, 2017), further investigations in nonhuman primates are merited.

This study investigates 2D:4D ratio in coppery titi monkeys (*Plecturocebus cupreus*; formerly *Callicebus cupreus*), an arboreal New World (NW) monkey species (Mason, 1966). To our knowledge only one study has assessed 2D:4D finger ratio in titi monkeys, and found no sex difference in 2D:4D ratio when ratios were averaged across the 13 NW species included in the study (one of which was titi monkeys; see Nelson & Shultz, 2010). This study was limited by several factors, including that the average 2D:4D ratio across many NW species may not reflect the patterns of individual species. Moreover, the sample sizes from most of the NW species in this study were relatively small[i] and perhaps underpowered to detect differences. Furthermore, the sample included NW monkeys from many institutions (at least seven), increasing the inter-measurer variability in 2D:4D measurement (differences in how technicians measure fingers can lead to measurement error; see Voracek, Manning, & Dressler, 2007). For these reasons, we investigated 2D:4D ratio in a single NW species using a larger sample and a closely controlled and uniform measurement protocol. We hypothesized that titi monkeys would exhibit a human-like pattern of sexually-dimorphic 2D:4D ratio and 2D:4D ratio asymmetry, with males exhibiting lower 2D:4D ratio and more right-biased (i.e., lower) 2D:4D ratio asymmetry than females.

A second purpose of this study was to investigate the relationship between maternal sex hormones during pregnancy and variation in offspring 2D:4D ratio. In humans, studies investigating direct measures of prenatal sex hormones and 2D:4D ratio have yielded mixed evidence (see Manning & Fink, 2017; Richards, 2017). For this reason, some have criticized 2D:4D ratio as an insensitive measure of prenatal sex hormone exposure (Richards, 2017; see also Breedlove, 2010). While 2D:4D ratio is not an exact proxy for prenatal sex hormone levels, it is safer and a better estimate of cumulative exposure than direct single-point

Nelson and Shultz (2010) collected data from 13 NW species, including howler monkeys, spider monkeys, titi monkeys, marmosets, tamarins, sakis, and squirrel monkeys. Most subjects were common marmosets (*Callithrix jacchus*, n = 76). The remaining subjects (*n* =131) came from 12 species (*Callicebus moloch*, also known as *Plecturocebus cupreus, n* = 32; *Aloutta caraya, n* = 22; all others, n < 10). See Table 1 in Nelson and Shultz (2010).

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measures of prenatal sex hormones from umbilical cord blood, amniotic fluid, or circulating maternal hormones during pregnancy (see Cohen-Bendahan, Van de Beek, & Berenbaum, 2005). Unlike 2D:4D ratio, direct hormone measures, obtained from a single time point during pregnancy, do not reflect the pooled effects of hormone exposure (for more detailed discussions of the validity of 2D:4D ratio, see Berenbaum, Bryk, Nowak, Quigley, & Moffat, 2009; Breedlove, 2010; Hönekopp & Watson, 2010; Manning, 2011; Richards, 2017). To our knowledge, no study has investigated whether direct measures of prenatal sex hormones correlate with 2D:4D ratio in nonhuman primates. In this study, we investigated the relationship between offspring 2D:4D ratio (measured when offspring were adults or sub-adults) and prenatal sex hormones in maternal urine samples obtained when the mother was pregnant. We hypothesized that titi monkeys exposed to high levels of maternal estrogen (as measured by urinary estrone conjugate (E_1C)) would exhibit a high (hypothesized to be female-typical) 2D:4D ratio and 2D:4D ratio asymmetry, and that exposure to high levels of maternal testosterone and testosterone-to-E_1C ratio would be associated with a low (hypothesized to be male-typical) 2D:4D ratio and a right-biased 2D:4D ratio asymmetry.

Methods

Subjects

Subjects were adult and sub-adult titi monkeys (mean age = 8.35 years old, *SD* = 5.76, range = [1–21]) housed indoors at the California National Primate Research Center (see Table 1 for a summary of descriptive statistics). Finger measurements and 2D:4D ratio data were obtained for *n* = 61 subjects (32 males, 29 females). Between these subjects there were 33 unique mothers. Most subjects (*n* = 44) had at least one sibling included in the study.

For a subset of *n* = 26 subjects (14 males, 12 females), maternal urine samples were assayed for testosterone and estrone conjugate (E_1C). Between these subjects there were 17 unique mothers. Most subjects in the subset (*n* = 14) had one sibling included in the study, and some subjects (*n* = 3) had two siblings (i.e., these three subjects were siblings with each other).

Subjects' housing and care procedures have been described previously (see Mendoza et al., 2015; Tardif et al., 2006). All adult subjects were housed with a pair-mate, and juvenile subjects were housed with their parents. All procedures in this study conformed to guidelines approved by the Institutional Association for the Care and Use of Animals (IACUC) at UC Davis, as well as national guidelines established in *The Guide for the Care and Use of Laboratory Animals* (National Research Council, 2010) and by the Animal Welfare Act.

Finger Measurements

Subjects' fingers were measured using the same procedures described in Baxter et al. (2018). Fingers were measured while subjects were sedated during semi-annual routine health assessments. Briefly, subjects were sedated following guidelines outlined in Tardif et al. (2006), with approximately 10 mg/kg ketamine, and monitored by trained animal care staff until they fully recovered from sedation.

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To measure fingers, one technician (the first author) depressed subjects' hands and fingers with a wooden craft stick, while another technician took direct measurements with a digital caliper. This was done because direct finger measurements with a caliper may yield more accurate and reliable measures than indirect measurements from photos or scans (Fink & Manning, 2018). Following guidelines set forth by Manning (2011), fingers were measured from the finger crease closest to the palm of the hand to the tip of the finger. Fingers were measured at least twice, until two measurements were obtained that were both within ±1.5 mm. The intra-class correlation coefficient for repeated finger measurements was high (for both the right and left hands, ICC = .93, $p < .001$). The two measurements that were closest in value were averaged to determine the finger length. Subjects' 2D:4D ratios for each hand were determined by dividing the average length of the second finger by the average length of the fourth. To determine asymmetry, subjects' left-hand 2D:4D ratio was subtracted from their right-hand 2D:4D ratio. In this study the same two technicians performed all finger measurements, which eliminated inter-technician measurement error (both technicians were trained to reliability > 88% agreement with other technicians in a previous investigation; see Baxter et al., 2018). Subjects' fingers were not measured if the subject was moving, or if they had previously-sustained damage on their second or fourth finger.

Maternal Urinary Sex Hormone Assay

Testosterone and E_1C concentrations were assayed from archived maternal urine samples. Details of urine collection (see Valeggia, Mendoza, Fernandez-Duque, Mason, & Lasley, 1999) and hormone assays have been described previously (Arias del Razo et al., in review; Jarcho, Mendoza, & Bales, 2012). Between 2007 and 2016, weekly maternal urine samples were collected between 0530–0630 hours (when the automatic overhead lights came on and the subjects first awoke), and were frozen at −80 °C within 20 minutes of collection until they were assayed. From the available sample bank, we selected maternal urine samples were selected that were obtained approximately during the 1st trimester of pregnancy (see Valeggia et al., 1999). Because exact conception dates were not known for all subjects and because titi monkey gestations typically last between 18–20 weeks (see Valeggia et al., 1999), the first trimester was conservatively defined as 15–20 weeks before the mother gave birth. The first trimester was chosen because studies in humans (Hickey et al., 2010) and rhesus monkeys (Abbott et al., 2012) suggest that developing fingers are more sensitive to the effects of prenatal sex hormones during early gestation, when compared to late gestation. Only subjects with a mother that had at least three available urine samples from the defined first trimester period were selected for maternal hormone analysis.

Urine samples were assayed using enzyme-immunoassays characterized for E_1C (Munro et al., 1991; Shideler et al., 1990) and testosterone (Arias del Razo et al., in review). Urine samples were diluted in water (1:200 ratio for E_1C, 1:20 ratio for testosterone), mixed with horseradish peroxidase conjugated to E_1C or androgen, and incubated overnight at 4°C in Nunc Maxisorb plates coated with antiserum, and read the next morning after plates were washed and 100 ul of freshly prepared substrate solution (0.05 M Citrate, pH 4.0, .4mM ABTS, 1.6mM H2O2) was added. Urinary creatinine concentrations were estimated using Taussky's methods (Taussky, 1954), and testosterone and E_1C are reported as ng/mg creatinine. Coefficients of variation for all assays were less than 15%. The ratio of maternal

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testosterone-to-E_1C was calculated by dividing testosterone by E_1C. For analyses, subject's average maternal testosterone, E_1C, and testosterone-to-E_1C ratio was used.

Control Variables: Age, Weight, Maternal Age, and Average Gestational Age at Sampling

Because some longitudinal studies in humans show that 2D:4D ratio increases slightly as children age (Trivers, Manning, & Jacobson, 2006; Wong & Hines, 2016), for analyses comparing sex differences in adult subjects' 2D:4D ratio, subjects' age at the time of finger measurement was considered. However, because age was not a significant covariate in any analysis, age is not included in the analyses reported (see the supplementary material for a summary of these analyses). Subjects' weight was not included in the analyses because it was highly correlated with subjects' sex (see Table 1) and age (for a summary of Pearson correlations between variables used in analyses, see Table 2 and Table 3).

As preliminary analyses showed that maternal age was negatively correlated with testosterone and testosterone-to-E_1C ratio, and that mothers that were further in their pregnancy had higher levels of testosterone and testosterone-to-E_1C ratio (see Table 3), all analyses were performed controlling for maternal age and offspring gestational age at time of sampling. Although all maternal urine samples were obtained from a specified period of time before birth during the first trimester (see above), some mothers had more urine samples available than others, resulting in slight variability in the average length of each offspring's gestation at the time of urine sampling. To control for gestational age, the number of days between the offspring's birth date and the date of each maternal urine sampling was calculated, and then averaged for each offspring. However, because neither maternal age nor average gestational age were significant covariates in any model with 2D:4D ratio, these variables are not included in the reported analyses (results from these models are available upon request).

Analyses

To test for sex differences in 2D:4D ratio, the t-tests that were performed as part of preliminary analyses (see Table 1) were repeated as linear mixed models (LMMs) to account for sibling pairs and other covariates (analyses were performed in R, using the "lmer" function from the "lme4" package; see Bates, Mächler, Bolker, & Walker, 2014). The dependent variables analyzed included subjects' right-hand 2D:4D ratio, left-hand 2D:4D ratio, and 2D:4D ratio asymmetry. For each dependent variable, analyses were performed with sex as the independent variable and Dam ID as a random effect. Each model was compared to a null model that only included Dam ID as a random effect with a log-likelihood test (Vuong, 1989). Because all models with significant effects of sex accounted for significantly more variance than the null model, we do not report these analyses in the main text (see supplementary material).

LMMs were also used to test associations between maternal sex hormones and offspring 2D:4D ratio. In each model Dam ID was included as a random effect. Separate analyses were performed for maternal E_1C, testosterone, and testosterone-to-E_1C ratio as independent variables. Each model was compared to a null model that only included Dam ID as a random effect with a log-likelihood test (Vuong, 1989). Because all models with significant effects

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of a maternal sex hormone accounted for significantly more variance than the null model, we do not report these analyses in the main text (see supplementary material). To assess whether associations between maternal sex hormones and offspring 2D:4D ratio were moderated by the offspring's sex, all analyses were repeated with offspring sex and the interaction between offspring sex and the maternal hormone included in the model. A log-likelihood test was used to determine whether this model accounted for more variance than the model with just the maternal sex hormone. Because none of the models were significantly improved by adding the offspring's sex, we do not report these analyses in the primary text (see supplementary material).

Results

Sex Differences in Right-hand 2D:4D Ratio

On average, males exhibited lower right-hand 2D:4D ratio when compared to females ($\beta = -0.29$, $SE = 0.13$, $t = -2.28$, $p = .023$; see Figure 1), a pattern similar to that seen in humans. Males' left-hand 2D:4D ratio did not significantly differ from females ($\beta = -0.18$, $SE = 0.14$, $t = -1.35$, $p = .18$), although the means were in the same direction as sex difference in right-hand 2D:4D ratio. Males' 2D:4D ratio asymmetry did not significantly differ from females ($\beta = -0.13$, $SE = 0.14$, $t = -0.96$, $p = .34$; see Figure 1).

Maternal E_1C Concentrations

Higher levels of maternal E_1C predicted lower offspring left-hand 2D:4D ratio ($\beta = -0.48$, $SE = 0.18$, $t = -2.60$, $p = .009$; see Figure 2). There was not an association between maternal E_1C and offspring's right-hand 2D:4D ratio ($\beta = -0.14$, $SE = 0.21$, $t = -0.66$, $p = .51$) or maternal E_1C and offspring's 2D:4D ratio asymmetry ($\beta = 0.30$, $SE = 0.21$, $t = 1.44$, $p = .15$; see Figure 2).

Maternal Testosterone Concentrations

Higher levels of maternal testosterone were associated with lower offspring right-hand 2D:4D ratio ($\beta = -0.53$, $SE = 0.19$, $t = -2.82$, $p = .005$; see Figure 2) and lower offspring 2D:4D ratio asymmetry ($\beta = -.43$, $SE = 0.20$, $t = -2.18$, $p = .03$). There was not an association between maternal testosterone and offspring's left-hand 2D:4D ratio ($\beta = 0.11$, $SE = 0.20$, $t = 0.57$, $p = .57$; see Figure 2).

Testosterone-to-E_1C Ratio

Higher levels of maternal testosterone-to- E_1C ratio were associated with lower offspring right-hand 2D:4D ratio ($\beta = -0.41$, $SE = 0.19$, $t = -2.20$, $p = .028$; see Figure 2) and lower offspring 2D:4D ratio asymmetry ($\beta = -.53$, $SE = 0.18$, $t = -2.95$, $p = .003$). There was not an association between maternal testosterone-to- E_1C ratio and offspring's left-hand 2D:4D ratio ($\beta = 0.37$, $SE = 0.20$, $t = 1.71$, $p = .09$).

Discussion

Results showed that titi monkeys exhibit a human- and ape-like pattern of sexually dimorphic 2D:4D ratio, with males exhibiting lower 2D:4D ratio than females on the right

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hand. To our knowledge, this is the first report of a significant sex difference in 2D:4D ratio in a NW primate species. It is also to our knowledge, the first to show that a NW monkey species shows a human-like 2D:4D pattern. These findings are particularly relevant because they show a prenatal hormone pattern consistent a prenatal organizational hypothesis. Specifically, maternal urinary sex hormones sampled during early gestation predicted the degree of offspring 2D:4D ratio, with high levels of maternal E_1C predicting the male-typical (low) left-hand 2D:4D ratio, and high levels of maternal urinary testosterone as well as maternal testosterone-to-E_1C ratio predicting male-typical (low) right-hand 2D:4D ratio and male-typical (low, right-biased) 2D:4D ratio asymmetry. While our results are correlational and cannot establish causation, they add to the growing literature suggesting that high levels of maternal sex hormones during early gestation may have a masculinizing effect on offspring 2D:4D ratio.

Sexual Dimorphism in Titi Monkeys' 2D:4D Ratio: Implications for Primate Phylogeny

As noted earlier, titi monkeys exhibited a similar pattern of sexually dimorphic 2D:4D ratio that is found in humans (Hönekopp & Watson, 2010) and great apes (McIntyre et al., 2009), with males exhibiting lower 2D:4D ratio than females on the right hand. Although prenatal sex hormones likely have organizational effects on both hands, our finding that sex differences are more robust for the right hand than the left hand is consistent with most research in humans (Hönekopp & Watson, 2010) and rhesus monkeys (Baxter et al., 2018) showing that the right hand exhibits greater sexual dimorphism than the left hand. To our knowledge only one other study has investigated 2D:4D ratio in titi monkeys and it failed to find a significant sex difference in 2D:4D ratio in the titi monkey sub-species that were investigated (Nelson & Shultz, 2010). However, as noted earlier, this earlier study was not specifically designed to assess sex differences in 2D:4D ratio in individual species, but instead averaged data across 13 different NW species (Nelson & Shultz, 2010). However, a recent study investigated 2D:4D ratio in a single NW species, neonatal marmosets (*Callithrix jacchus*), and the study showed no sex differences between infants (Frye, Rapaport, Melber, Sears, & Tardif, 2019). One other study, however, did find evidence that another NW species shows a similar digit ratio pattern to humans, great apes, and to the pattern we found in titi monkeys (Hart, 2018). While not directly measuring finger ratio, the researcher measured metacarpal ratio length in howler monkeys (*Aloutta seniculus*) and owl monkeys (*Aotus azarae*), and found that in owl monkeys, males exhibited a lower second-to-fourth metacarpal length ratio than females (Hart, 2018). However, it should be noted that metacarpal length ratios may not be directly comparable with finger length ratios (see McFadden & Bracht, 2003; McFadden & Bracht, 2005), and hence, further research using a larger and more homogeneous species sample of finger ratios is needed to investigate whether other NW monkeys exhibit the human and titi monkey pattern of sexually dimorphic 2D:4D finger ratio.

In phylogenetic context, our findings demonstrate that the reversed pattern of 2D:4D ratio observed in several OW monkey species (males exhibiting higher ratio than females; see Abbott et al., 2012; Baxter et al., 2018; Roney et al., 2004) is not present in all monkey species. It is possible that patterns of sexually dimorphic 2D:4D ratio vary across primate super-families (for a visual representation of 2D:4D ratio across primate phylogeny, see

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Figure 3). To the extent that the titi and owl monkeys represent a common NW pattern, it is possible that the NW monkey pattern of 2D:4D ratio was conserved in a common ancestor. Before concluding this, however, it would be necessary to sample a variety of other NW species, particularly given that the Callicebus and Aotus are more closely related than most other *Platyrrhini*. Consistent with this, in other studies that included NW species, researchers failed to detect a sexually-dimorphic 2D:4D ratio (Hart, 2018; Nelson & Shultz, 2010). Owl and titi monkeys are, on average, smaller, more arboreal, are capable of forming adult pair bonds, and are possibly less likely to demonstrate tool use when compared to OW and most other NW species (Fernandez-Duque, Rotundo, & Sloan, 2001; Terborgh & Janson, 1986). Additionally, the owl monkey is the only nocturnal NW species of primate. The general differences between these two species and other NW monkeys may point to differential selective pressures. If this is true, the pattern of digit ratio observed in these species might differ when they are compared to other NW species, and the titi and owl monkey patterns may not be universal to all NW species. Further investigation of 2D:4D ratio in much larger number of primate species is needed to explore the unique vs common niches that might have selected for patterns of 2D:4D ratio and whether different sexually-dimorphic patterns of 2D:4D ratio are due to different patterns of androgen and estrogen receptor expression in the fingers.

Offspring 2D:4D Ratio is Associated with Prenatal Maternal Urinary Sex Hormones

We found that low 2D:4D ratio (male-typical, for titi monkeys) in the offspring was associated with high levels of maternal urinary E_1C, testosterone, and testosterone-to-E_1C. For maternal E_1C, this association was found only with left-hand 2D:4D ratio, and for maternal testosterone and testosterone-to-E_1C hormone ratio, the associations were found only with right-hand 2D:4D ratio. The strength of these associations between 1st trimester maternal hormones and offspring 2D:4D ratio (effect sizes for significant associations ranged from −0.41 to −0.53) are approximately twice as large as the effect sizes reported in studies of 2nd trimester maternal hormones and offspring 2D:4D ratio in humans (see Barona et al., 2015; Richards et al., 2018; Ventura et al., 2013). Given that the technicians that measured fingers in this study had the high inter-rater reliability and long-term experience with measuring the right and left hand, we do not believe this is measurement error between the hands. Corroborating this belief, these findings are consistent with a meta-analysis of human studies showing that sexual dimorphism is more consistent for the right-hand 2D:4D ratio than for left-hand 2D:4D ratio (Hönekopp & Watson, 2010), as well as several other studies directly measuring the relationship between prenatal hormones and 2D:4D ratio. In many studies, direct measures of prenatal sex hormones predicted 2D:4D ratio on one hand but not on the other (see Barona, Kothari, Skuse, & Micali, 2015; Hollier et al., 2015; Lutchmaya et al., 2004; Ventura, Gomes, Pita, Neto, & Taylor, 2013; Whitehouse et al., 2015). Although further research is needed to understand differential associations between prenatal sex hormones and right-hand vs left-hand 2D:4D ratio, our finding that prenatal testosterone was linked more strongly with right-hand 2D:4D ratio than with left-hand 2D:4D ratio is not surprising, given that right-biased lateralization may be associated with masculinization (see Manning, 2002; Tanner, 1990). This is also congruent with our finding that that low (right-biased) 2D:4D ratio asymmetry was associated with high levels of maternal testosterone and testosterone-to-E_1C hormone ratio.

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Concerning testosterone and testosterone-to-E_1C ratio, our findings are consistent with several other studies showing a negative correlation between 2D:4D ratio and prenatal sex hormones, including two studies of circulating testosterone in pregnant females' plasma (Barona et al., 2015; Ventura et al., 2013) (however, see Hickey et al., 2010), one study of testosterone in amniotic fluid (Richards, Gomes, & Ventura, 2018; Ventura et al., 2013), one study of testosterone-to-estrogen ratio in amniotic fluid (Lutchmaya et al., 2004), and three studies of androgens in postnatal umbilical cord blood (Mitsui et al., 2016; Mitsui et al., 2015; Whitehouse et al., 2015). Our findings that testosterone and testosterone-to-E_1C ratio were negatively correlated with 2D:4D ratio asymmetry is consistent with the view that low (right-biased) 2D:4D ratio asymmetry reflects higher levels of prenatal masculinization (see Manning, 2002). To our knowledge, only one other study has investigated prenatal sex hormones and offspring 2D:4D ratio asymmetry, and found no association between offspring asymmetry and androgens assayed from 2nd or 3rd trimester maternal plasma (Hickey et al., 2010). Hence, the results of the current study are the first report of a significant association between directly measured prenatal sex hormones and offspring 2D:4D ratio asymmetry, and add to the growing literature showing an association between prenatal maternal testosterone (alone and in proportion to maternal estrogen) and 2D:4D ratio (as well as 2D:4D ratio asymmetry). While cause and effect cannot be established from this correlational study, one interpretation is that prenatal testosterone has an organizational masculinizing effect on offspring 2D:4D ratio that leads to differential finger ratio. Our findings add to growing evidence suggesting that 2D:4D finger ratio is a valid estimate of prenatal testosterone exposure.

While the relationship between testosterone and 2D:4D ratio was in the expected direction, one unexpected finding was that the negative correlation between maternal E_1C and offspring 2D:4D ratio was not as hypothesized. Moreover, this relationship was the only one with the left hand. If this finding replicates, one possible explanation is that the negative correlation between maternal E_1C and offspring 2D:4D ratio may reflect the secondary effects of androgens aromatized into estrogens (see Gill, Hosking, & Egan, 1998; White, 2006), potentially androstenedione, from which estrone is aromatized (Gruber, Tschugguel, Schneeberger, & Huber, 2002). In other words, high levels of maternal E_1C in urine may be a proxy for high levels of maternal androgens, as aromatization may lead high levels of androgens to be converted to high levels of estrogens (see Simpson, 2002). Because prenatal androgens masculinize 2D:4D ratio, this could explain why high levels of maternal E_1C were associated with low offspring 2D:4D, a finding consistent with one study in humans that found high levels of prenatal estrogen (measured in umbilical cord blood) were correlated with low (male-typical, for humans) 2D:4D ratio in male infants (Hollier et al., 2015). However, without direct measures of aromatization, this explanation remains speculative. Further research is needed to understand how prenatal sex hormones affect 2D:4D ratio, especially using methods that account for aromatization.

Conclusions

This study is the first to show a sex difference in 2D:4D finger-length ratio in a NW primate species. Paralleling research in humans, male titi monkeys exhibited lower right-hand 2D:4D ratio when compared to females. In the context of studies investigating 2D:4D ratio in other

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nonhuman primate species, this finding demonstrates that in humans (Hönekopp & Watson, 2010), apes (McIntyre et al., 2009), and at least one species of NW monkeys, the same pattern of sexually-dimorphic 2D:4D finger ratio is observed. This stands in contrast to some OW monkeys that exhibit a reversed pattern of 2D:4D ratio (Abbott et al., 2012; Baxter et al., 2018; Roney et al., 2004). Further research is needed with an increased number of NW species, larger sample sizes, and consistent methods to measure finger ratio. This is also the first study to show a correlation between maternal sex hormone levels during pregnancy and offspring 2D:4D ratio in a nonhuman primate species. Maternal E_1C, testosterone, and testosterone- E_1C ratio predicted lower (male-typical, for titi monkeys) 2D:4D ratio, and maternal testosterone and testosterone- E_1C ratio predicted lower (right-biased and male-typical in humans) 2D:4D ratio asymmetry. Although further research is needed to understand how maternal circulating hormones affect the developing fetus, our results suggest that maternal sex hormones may have a masculinizing effect on 2D:4D ratio. Although studies of prenatal maternal sex hormones in humans have primarily investigated plasma, our study demonstrates that maternal urine is also a valid medium. Because maternal urine is non-invasive and can be easily sampled at multiple time points to provide a pooled estimate of long-term hormonal exposure during pregnancy, maternal urine may be a useful medium for future investigations of maternal sex hormones during pregnancy in humans. Moreover, maternal urine is a safe to collect during the first trimester, when prenatal sex hormones likely have the strongest organizational effects on 2D:4D ratio (see Abbott et al., 2012; Manning & Fink, 2017). Together, our findings provide important evidence that 2D:4D ratio is a valid biomarker for prenatal sex hormone exposure in titi monkeys. Furthermore, because titi monkeys, like humans, are one of the few mammalian species that form monogamous pair-bonded relationships (Bales et al., 2017; Kleiman, 1977), future investigation of titi monkeys' 2D:4D ratio may shed light on how prenatal sex hormones contribute to social behavior in close relationships.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author and will be made available to any qualified researcher.

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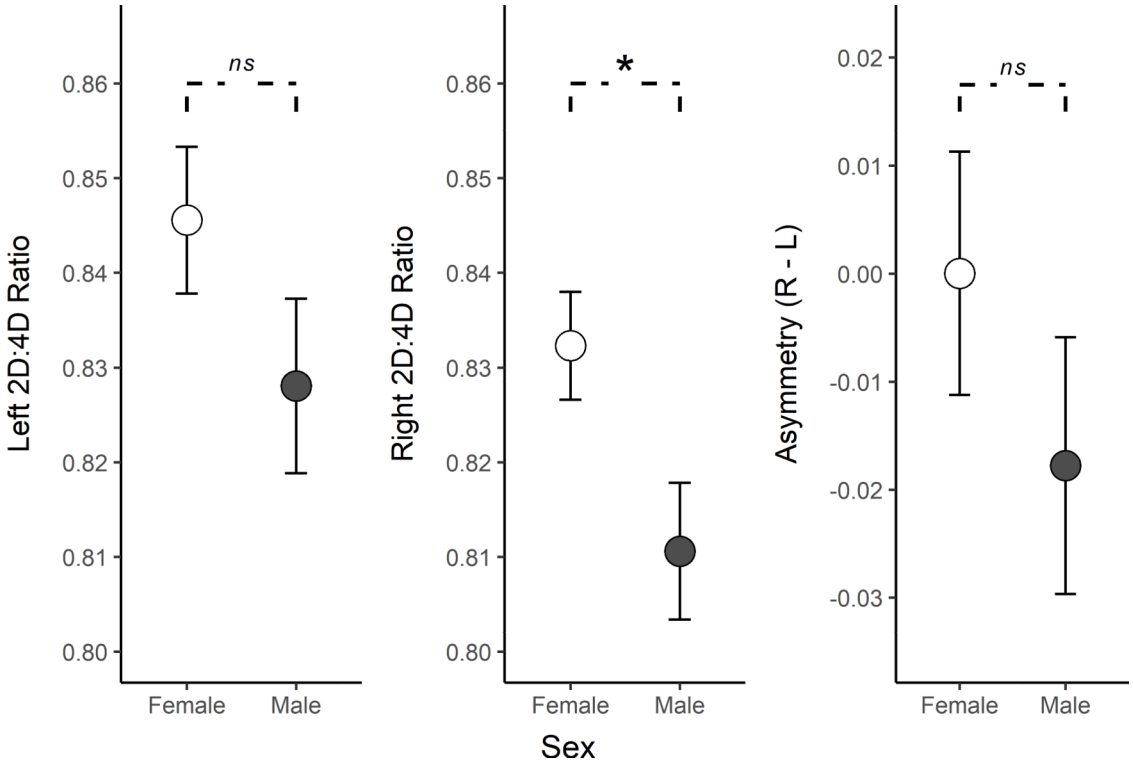

Figure 1. Sex Difference in Titi Monkeys' Right-hand 2D:4D Ratio.

Description: The graphs show titi monkeys' left-hand 2D:4D ratio, right-hand 2D:4D ratio, and 2D:4D ratio asymmetry (right-hand – left-hand) by sex. The x-axis represents subjects' sex, and the y-axis represents 2D:4D ratio (the left and middle panels) or 2D:4D ratio asymmetry (the right panel). The white circles represent the mean for females, the black circles represent the mean for males, and the error bars represent ±1 standard error. The * with dashed lines indicates a significant comparison at $p < 0.05$, and the ns with dashed lines indicates the difference between means is not significant ($p > 0.05$).

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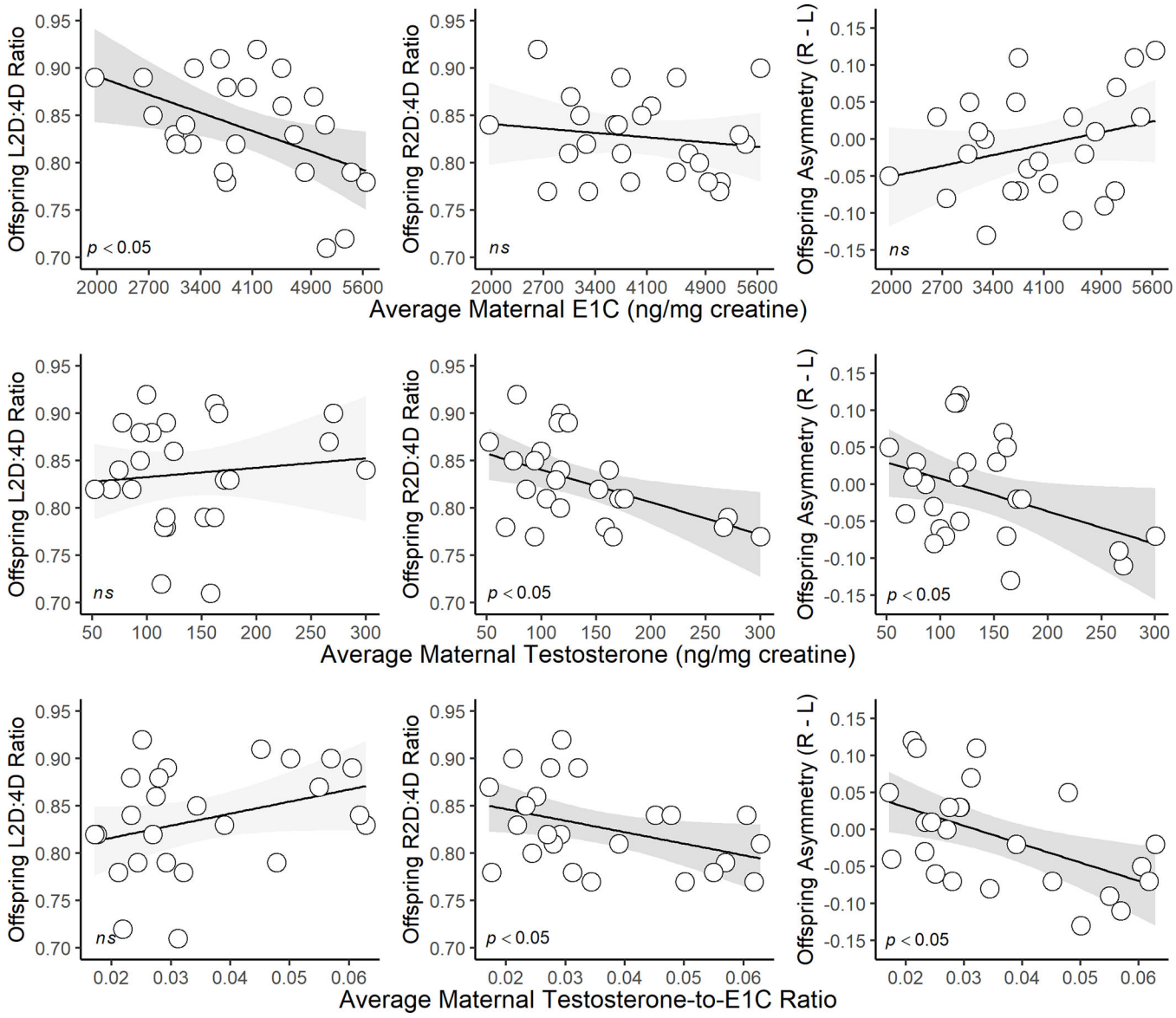
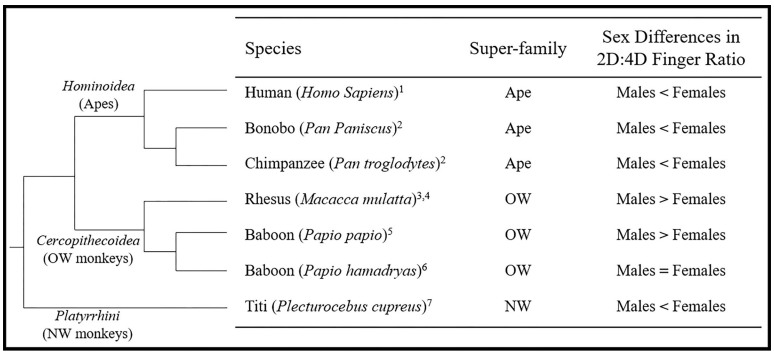


Figure 2. Maternal Sex Hormones are Associated with Offspring 2D:4D Ratio Differentially Across the Hands.

Description: The graph depicts the association between maternal urinary sex hormones during pregnancy and offspring left-hand 2D:4D ratio, right-hand 2D:4D ratio, and 2D:4D ratio asymmetry (right-hand – left-hand) measured in adulthood. In the left and middle column panels, the y-axis depicts offspring 2D:4D ratio; in the right column panels, the y-axis depicts offspring 2D:4D ratio asymmetry. In all graphs, the solid black line represents the line of best fit and the shaded gray region represented the standard error of prediction. In the bottom left-hand corner of each graph, $p < 0.05$ indicates the association is significant, and ns indicates the association is not significant ($p > 0.05$).



Species	Super-family	Sex Differences in 2D:4D Finger Ratio
Human (*Homo Sapiens*)[1]	Ape	Males < Females
Bonobo (*Pan Paniscus*)[2]	Ape	Males < Females
Chimpanzee (*Pan troglodytes*)[2]	Ape	Males < Females
Rhesus (*Macacca mulatta*)[3,4]	OW	Males > Females
Baboon (*Papio papio*)[5]	OW	Males > Females
Baboon (*Papio hamadryas*)[6]	OW	Males = Females
Titi (*Plecturocebus cupreus*)[7]	NW	Males < Females

Figure 3. Sex Differences in Primates' 2D:4D Ratio Exhibits Phylogenetic Variation Across Primate Superfamilies.

Description: The figure depicts the direction of sex differences in 2D:4D ratio across primate phylogeny. The tree is based on commonly accepted phylogenetic estimates (Arnold, Matthews, & Nunn, 2010; Perelman et al., 2011). Abbreviations: OW indicates Old World; NW indicates New World; Males < Females indicates that, on average, males exhibit lower 2D:4D ratio than females; Males > Females indicates that on average, males exhibit higher 2D:4D ratio than females; Males = Females indicates no sex differences observed in 2D:4D ratio. For a note on studies omitted from this table, see the supplementary material.

References in Figure: Hönekopp & Watson (2010) (among many others); 2 McIntyre et al. (2009); 3 Baxter et al. (2018); 4 Abbott et al. (2012); 5 Roney et al. (2004); 6 Huber et al. (2017); 7 The Current Study

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Table 1.

Descriptive Statistics of Study Variables.

	Total		Males		Females		Comparison			
Variable	**Mean**	***SD***	**Mean**	***SD***	**Mean**	***SD***	***DF***	***t***	***p***	***d***
All Subjects										
R2D:4D Ratio	0.82	0.04	0.81	0.04	0.83	0.03	54	2.25	0.03*	0.60
L2D:4D Ratio	0.84	0.05	0.83	0.05	0.85	0.04	53	1.35	0.18	0.37
Asymmetry (R - L)	−0.01	0.06	−0.02	0.07	0.00†	0.06	50	1.00	0.32	0.28
Age (years)	8.42	5.78	8.33	6.02	8.52	5.60	59	0.13	0.90	0.03
Weight (kg)	1.19	0.19	1.27	0.19	1.11	0.15	59	−3.64	<.001*	0.93
Subset of Subjects with Maternal Urinary Data										
Testosterone (ng/mg creatinine)	138	62	143	69	131	56	24	−0.49	0.63	0.19
E1C (ng/mg creatinine)	4,008	975	4,140	873	3,866	1,095	23‡	−1.11	0.28	0.44
T-E_1C Ratio	0.04	0.01	0.03	0.01	0.04	0.02	24	0.38	0.71	0.15
Dam Age at Sample (years)	7.5	4.0	8.4	4.6	6.3	3.1	24	−1.34	0.19	0.53
Gestational Age (days)	87.4	3.4	87.5	2.7	87.3	4.2	24	0.19	0.89	0.08

Description:

*indicates $p < .05$

†The value females' average digit asymmetry (r-l) is $3.25e^{-5}$ and was rounded for display in the table

‡One high outlier was excluded from the analysis of E1C. Because exact conception dates were not known for all subjects, Gestational Age was calculated relative to days-before-birth (see text for more information). The degrees of freedom for the t-tests comparing sex differences in R2D:4D ratio, L2D:4D ratio, and digit ratio asymmetry are not equal to 59 because some subjects were missing data for one hand but were not missing data for the other hand.

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Table 2.

Pearson Correlations Between 2D:4D Ratio, Age, and Weight.

Variable	1. L2D:4D	2. R2D:4D	3. Age
All Subjects			
1. L2D:4D Ratio	–		
2. R2D:4D Ratio	.03	–	
3. Age	–.14	–.21	–
4. Weight	–.38*	–.11	.33*
Subset of Subjects with Maternal Urinary Data			
1. L2D:4D Ratio	–		
2. R2D:4D Ratio	.02	–	
3. Age	–.20	–.005	–
4. Weight	–.23	–.04	.71*

Description:

* indicates p < . 05 *L2D:4D* indicates left-hand 2D:4D ratio. *R2D:4D* indicates right-hand 2D:4D ratio.

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Table 3.

Pearson Correlations Between Maternal Sex Hormones and Maternal Demographics.

Variable	1. Testosterone	2. E_1C	3. T- E_1C Ratio	4. Dam Age
1. Testosterone (ng/mg creatinine)	–			
2. E_1C (ng/mg creatinine)	.37 †	–		
3. T- E_1C Ratio	.76 *	−.25	–	
4. Dam Age at Sample	−.01	.09	−.09	–
5. Gestational Age	.45 *	.12	.41 *	−.31

Description:

* indicates $p < .05$

† indicates $.05 < p < .10$.

E_1C indicates estrone conjugate; *T- E_1C Ratio* indicates testosterone-estrone conjugate ratio (calculated by dividing testosterone by estrone conjugate). Because exact conception dates were not known for all subjects, Gestational Age was calculated relative to days-before-birth (see text for more information).