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Publication Date

2022-06-01

DOI

10.1016/j.yhbeh.2022.105157

Peer reviewed

HHS Public Access

Author manuscript

Horm Behav. Author manuscript; available in PMC 2023 June 01.

Published in final edited form as:

Horm Behav. 2022 June ; 142: 105157. doi:10.1016/j.yhbeh.2022.105157.

The Onset of Puberty in Colony-housed Male and Female Titi Monkeys (*Plecturocebus cupreus*): Possible Effects of Oxytocin Treatment during Peri-adolescent Development

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Abstract

Oxytocin has been used to treat neurodevelopmental conditions in adolescent patients but possible effects on reproductive development have not been well investigated. The effects of daily intra-nasal oxytocin treatment (12–18 months of age) on puberty and fertility were studied in colony-housed, male and female titi monkeys (*Plecturocebus cupreus*). Body weight, urinary conjugated pregnanes and estrogens (defining cyclicity) in females, and androgens and sperm in urine of in males, were measured from 1–3 years of age to detect puberty. Serum testosterone was also measured in males at 13, 23 and 33 months of age and hemi-castration at 3 years of age enabled assessment of testicular morphometry and oxytocin receptor expression. An oxytocin treatment*time interaction suggested a minor, transient suppression in weight gain after treatment ended. Note that females weighed 10% less across all ages. Oxytocin-treated females exhibited early, spurious ovulations but neither regular cyclicity (≈30 months) nor pregnancies were affected by treatment. Oxytocin did not affect the pubertal increase in urinary androgen or the first appearance of sperm, which occurred as early as 15 months of age. Treatment did delay the puberty-associated rise in serum testosterone in males. All males were pubertal by 22 months and all females by 32 months of age. Although no major male or female fertility outcome was observed, oxytocin demonstrated some physiological effects through a delay of testosterone secretion in males, induction of precocious ovulation in females, and a suppression of general weight gain for the months following treatment.

Keywords

Primate; puberty; oxytocin; cyclicity; ovulation; spermatogenesis

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Declaration of interest: The authors have no competing interests to declare.

Introduction

Puberty is a complex developmental process, the progression of which is not easy to define or assess with precision, in part because it results cumulatively from morphological, physiological and behavioral events that collectively culminate in sexual maturity and fertility (Rowell 1977, Ojeda, Andrews et al. 1980, Plant, Terasawa et al. 2015). Hormones of the reproductive axis play a critical role in initiating and directing the process, and the transition which occurs during adolescence represents a period of particular psychosocial and developmental plasticity (Viner, Allen et al. 2017). Oxytocin is one such hormone released from the posterior pituitary gland during parturition, milk letdown and intercourse; as well as throughout the brain, potentially influencing social and non-social behaviors (Carter 1998, Lee, Macbeth et al. 2009). As a result, oxytocin and related compounds have been used to treat neurodevelopmental conditions in adolescent patients including autism spectrum disorders (Anagnostou, Soorya et al. 2014) and Prader-Willi syndrome (Dykens, Miller et al. 2018). Our laboratory has conducted studies investigating the potential effects of daily intra-nasal oxytocin treatment of peri-adolescent male and female titi monkeys on their affiliative behaviors as adults (Arias Del Razo, Berger et al. 2020). However, results of studies in male rodents, both *in vivo* (Ang, Ungefroren et al. 1991, Nicholson, Guldenaar et al. 1991, Bales, Abdelnabi et al. 2004, Bales, Perkeybile et al. 2012) and *in vitro* (Adashi and Hsueh 1981, Kwan and Gower 1988), indicate that oxytocin can diminish testosterone synthesis, the expression of sexual behavior and thereby reproductive potential in males. Oxytocin may also be involved in regulating the onset of female cyclicity and reproductive success (Withuhn, Kramer et al. 2003, Cushing, Levine et al. 2005). To date, there has been relatively little attention given to the possible effects of oxytocin treatment on reproductive development or post-pubertal fertility in primates.

Non-human primates are valuable scientific, biomedical and experimental models, sharing physiological, neuroanatomical and reproductive, as well as developmental, cognitive and social similarities with humans (Phillips, Bales et al. 2014). Social cohesion, and the significance of social monogamy, are topics of considerable current interest, contributing to reproductive fitness in many species (Thompson 2019). However it is defined, monogamy is more common among primates than other mammalian taxa (Diaz-Munoz and Bales 2016, French, Cavanaugh et al. 2018). Still, even among primates, stable family units supported by monogamy as a social construct are not seen in a majority of species perhaps because monogamy incurs costs not expected to favor evolutionary success (Carter and Perkeybile 2018, Klug 2018). Titi monkeys, as a socially monogamous primate, provide a promising model for human pair bonding, the psychosocial wellbeing that is enhanced by it and the potential effects of oxytocin on these traits (Tardif, Bales et al. 2006, Bales, Arias Del Razo et al. 2017, Savidge and Bales 2020). Moreover, relatively little is known of the reproductive biology of titi monkeys (Valeggia, Mendoza et al. 1999). No studies specifically investigating reproductive development have been reported for either males or females of this species. Whether or not oxytocin treatment during juvenile or adolescent development might have detrimental effects on the reproductive success of this monogamous primate has not been examined.

Based on observations in rodents, we hypothesized that oxytocin treatment would delay sexual maturation and decrease fertility. To test that hypothesis, male and female titi monkeys were treated daily with saline or oxytocin intra-nasally from 12 to 18 months of age (Arias Del Razo, Berger et al. 2020). Reproductive function was monitored in both sexes from approximately 1 to 3 years of age using urinary steroid hormone concentrations. The appearance of sperm in urine or in stimulated ejaculates of males as well as serum testosterone concentrations (at 13, 23 and 33 months of age) were also examined. Testicular tissue was recovered by hemi-castration performed at 3 years of age for morphometric and gene expression analysis. Puberty was defined by regular, cyclic elevations of immuno-reactive pregnanes and estrogen conjugates (Lasley, Stabenfeldt et al. 1985, Valeggia, Mendoza et al. 1999) as evidence of ovulation in females, and the appearance of sperm in males (urine or ejaculate). Fertility was assessed by the establishment of pregnancies after pairing males and females with reproductively experienced, fertile mates.

Materials and Methods

Subjects: Female and male titi monkeys were enrolled in a study reported previously investigating the possible effects of intra-nasal oxytocin administration (n=8 females, n=7 males) or saline (n=7 females, n=7 males), given daily from 12 – 18 months of age, on reproductive development and behavior (Arias Del Razo, Berger et al. 2020). All protocols and procedures used in the conduct of the research as described were approved by the Institutional Animal Care and Use Committee, University of California, Davis. Birth weights were measured and body weights were monitored at least once a week from the start of the study period (≈12 months of age). Oxytocin acetate salt (Santa Cruz Biotechnology, Dallas, Texas, USA) was dissolved in saline so as to administer 0.8 IU/kg of body weight (0.8IU/kg =1.6 μg/kg) by intra-nasal application each day using a pipette dripping 25 μl of oxytocin solution or saline into each nostril. Subjects were trained using positive reinforcement techniques and were habituated to all required steps before treatments began. Sampling of urine started at 12 months of age and continued three times a week on average through peri-adolescent development until pregnancies were achieved in subject females paired with (known fertile) mature males or subject males established a pregnancy with a mature (known fertile) female partner. Subjects were removed from their family units and paired at 898 ± 3 days (≈30 months) of age, with mature, unrelated partners of the opposite sex. Procedures involving anesthesia [for positron emission tomography (Arias Del Razo, Berger et al. 2020)] were performed at 13, 23 and 33 months which allowed blood samples to be collected from the saphenous vein for analysis of testosterone in males. Males were also conditioned to mechanical stimulation of their perineal region with a vibrator to stimulate ejaculation.

Testicular analysis: Males underwent hemi-orchidectomy at 36 months of age. Testis was weighed and testicular tissue was frozen for transcript analysis as well as fixed for morphometric analysis after immuno-histochemistry. The forward and reverse primers for the reference gene HPRT (ATGCTGAGGATTTGGAAAGGGT and AGAGGGCTACAATGTGATGGC, respectively) yielded a product size of 114 bp, and at 50 nM primer concentration, had an efficiency of 104.8%. The forward and reverse primers for the oxytocin receptor (CAAGATCCGCACGGTCAAG and

CGAAGAAGAAAGGCGTCCAG, respectively) amplified a 74 bp product with 97% efficiency at 300 nM. Sertoli cell numbers were determined following immuno-labeling of GATA4 as previously described (Berger, Sidhu et al. 2019). Numbers of initial spermatogonia were similarly determined using immuno-labeling with UTF1 (Kristensen, Nielsen et al. 2008). Detergent resistant spermatid numbers, a measure of spermatogenic efficiency, were determined in testicular tissue from four males (Amann and Lambiase 1969).

Steroid analysis: Urine collections were conducted from inside the cage between 5:45 and 7:00 AM using disposable specimen cups, and samples were stored in aliquots at −80°C. Assays for urinary estrogen (E1C) and pregnanediol (PgG) conjugates were conducted essentially as previously described (Valeggia, Mendoza et al. 1999) using the same primary antisera (Clinical Endocrinology Laboratory, University of California, Davis). Briefly, urine was diluted in water to ensure determinations were within the range of the standard curves for each analyte. For estrogen analysis, urine samples from cyclic females were diluted 1:200 and samples from pregnant females were diluted 1:1000, 40 μL of which was assayed in each case. For pregnanes, samples were diluted 1:4 with water, 20μL of which was assayed. Nunc Maxisorb plates were coated with antisera (R522–2 for E1C or R13904 for PdG, both at 1:10,000 in coating buffer) and washed before adding standards and samples. The standard curve for E1C ranged from 0.6 – 300 pg/well, and for PdG from 0.8 – 10 pg/well. A polyclonal antisera (R156/7) raised against testosterone (Clinical Endocrinology Laboratory, University of California, Davis) that has been used previously in reproductive monitoring of fecal androgen analysis (Bales, French et al. 2006) was used for the analysis of urine from males. Since the possible conjugated metabolites of testosterone and other androgens in urine (Hagey and Czekala 2003) have not been characterized in titi monkeys, the results are described hereafter as urinary androgens. For urinary androgen determinations, 500 μl of urine was hydrolyzed with 100 μl of HCl, diluted 1:21 (25 μl into 500 μl of buffer), 50 μl of which was assayed directly. Standard curve was 1.95–1000 pg/well. Horseradish peroxidase (HRP) conjugated to E1C (1:240,000 dilution) or PdG (1:150,000 dilution) was added, plates were thoroughly mixed and incubated at 4°C overnight. The following morning, plates are washed 4 times in wash solution, then 100 ul of freshly prepared substrate solution (0.05 M citrate, pH 4.0, .4 mM ABTS, 1.6mM H_2O_2) was added. Plates were read when the average optical density (OD) of the total binding wells was at an absorbance of 1.0. Urinary steroid concentrations were expressed after normalization to creatinine concentrations using established methods (Taussky 1954). Intra- and inter-assay coefficients of variation were <15% for both E1C and PdG assays. Testosterone concentrations were also determined in serum samples from males by radio-immunoassay, essentially as reported previously (Stabenfeldt, Hughes et al. 1979). Briefly, 50 μL of serum was extracted with diethyl ether, dried, reconstituted in buffer and incubated overnight with diluted antisera (S-250, Dr. G. Niswender, 1:130,000) and tracer (1,2,6,7-^{3}H-testosterone). Free tracer was separated using dextran-coated charcoal and the bound fraction counted after addition of scintillation cocktail. The standard curve ranged from 2.5–200 pg/tube. All samples were assayed in a single assay and the intra-assay coefficient of variation was <10%.

Statistical analysis

Body weight was not normally distributed and was consequently log transformed to meet assumptions of ANOVA. Data were analyzed with individual nested within main effects of sex and treatment, both considered fixed factors, and age as the repeated measure using the aov procedure in R. Correlations between birth weight and body weight at 12 mo of age were analyzed separately for males and females using cor.test procedure in R. Plasma testosterone concentrations were not normally distributed and were log transformed before analysis to meet both normality and homogeneity of variance assumptions for ANOVA. The log10 transformed testosterone data at each age, testis weights, Sertoli cell numbers, spermatogonial cell numbers and oxytocin receptor transcript concentration in testis and vas were analyzed using the lmer procedure in R with individual males as random factor and treatment as a fixed factor. Effect size (η^2_p) was calculated as: Sums of squares of effect/ [Sums of squares of effect + Sums of squares of error] (Lakens 2013). Since partial effect size is equal to effect size for one-way ANOVA, only η^2 is indicated in this circumstance. A P value of 0.05 was accepted as indicative of statistical significance.

Results

Females and males averaged 0.078 ± 0.003 and 0.086 ± 0.012kgs at birth and by 12 months of age were 0.808 ± 0.017 and 0.893 ± 0.012kgs, respectively. There was a significant positive correlation between weight at birth and at 12 months of age among females (R^2 = 0.46, P < 0.01) but not in males (R^2 = 0.17, P > 0.05). Males were heavier on average than females at all ages thereafter (Figure 1). Male weights appeared to plateau briefly around 18–20 months of age, but accelerated again from 24–30 months of age. Female body weights increased more consistently month to month until their growth began to slow around 30 months of age (Figure 1). Body weight was significantly affected by age ($F_{1,689}$ 3933, η^2_p = 0.85, P < 0.0001) and sex ($F_{1,23}$ 26.70, η^2_p = 0.54, P < 0.0001) but there was no main effect of oxytocin treatment. There were significant treatment by age ($F_{1,689}$ 5.342, η^2_p = 0.008, P = 0.02) and sex by age interactions ($F_{1,689}$ 25.688, η^2_p = 0.04, P < 0.0001) but no significant treatment by sex interaction. The decrement in female compared with male body weight (-10%) remained relatively constant throughout.

The initiation of ovarian activity was evident by increases in urinary steroid secretion after an initial period of consistently low E1C (<500ng/mg Cr) and low PdG (<100ng/mg Cr) concentrations in females from 12 months until at least 20 months of age (Figure 2). Ovulation was assumed to have occurred if PdG concentrations were >100ng/mg Cr in two consecutive samples that together totaled >400ng/mg Cr, and were defined as luteal phases. E1C concentrations were higher than PdG. Elevations in urinary PdG indicative of luteal phases coincided with elevations in E1C and occasionally a second elevation was observed that immediately preceded the initiation of the luteal phase and presumptive ovulation. Regular cyclicity was defined by 3 or more such luteal phases occurring at intervals averaging 17–18 days. Using these criteria, regular cyclicity was established at 924 ± 10 days (2.5 years) of age (Figure 2A–D). In several females, much earlier, luteal phases were observed, varying in number (1–6), and occurring at irregular intervals and with variable peak PdG concentrations (range 235–2468 ng/mg Cr, Figures 2B and C). Five

of the six females that experienced the earliest luteal phases were treated with oxytocin and oxytocin-treated females experienced their first luteal phases earlier than saline-treated females (717 ± 40.4 vs 868 ± 40.4, respectively, P=0.02). These early luteal phases were followed by variable periods of apparent ovarian inactivity (Figure 2B & C) until sustained cyclicity was initiated.

Females were paired with males at 898 ± 3 days of age. Some females had initiated cyclicity before introduction of males (Figure 2A) and the introduction of a male did not appear to have an immediate effect on cyclicity in those that had not. Pregnancy was associated with sustained and increasingly elevated PdG and E1C concentrations exceeding the length of normal luteal phases (Figures 2A–D). First pregnancies were established (by sexually mature male partners) in subject females at 1113 ± 70 days (≈3 years) of age and proceeded to term (133.6 ± 1.3 days of gestation, n=8; Figure 2A–C) or ended in a miscarriage at 76.3±13.3 days (range 33–117 days; saline treated n=4, oxytocin treated n=3; Figure 2D). The loss of pregnancy was characterized by the appearance of blood around the genitalia in three females at 41, 47 and 117 days of gestation. These lost pregnancies were confirmed later to be coincident with an abrupt decline in PdG and E1C concentrations in urine (Figure 2D) and with the resumption of cyclicity thereafter. The other four pregnancy losses were identified only by the sudden and precipitous disappearance of urinary PdG and E1C. These miscarriages were not associated with any obvious excretory signature of either steroid conjugate that was apparent before or after pregnancy was established or prior to the loss of the pregnancy.

Urinary androgen concentrations increased progressively and in a linear fashion from 12 to 24 months of age in both saline and oxytocin-treated males (Figure 3). Sperm (Figure 3, insert) was first detected in urine, or in ejaculates when these were obtained, as early as 15 months of age and from all males by 22 months of age. Serum testosterone increased with age in control males (0.40 ± 0.70, 1.99 ± 0.81 and 2.29 ± 1.46 ng/ml at 13, 23 and 33 months of age, respectively; Table 1, $F_{2,12}$ 5.93, $\eta^2_p = 0.50$, P = 0.016). There was considerable variability among individuals, especially at months 13 (oxytocin-treated; range 0.26 – 4.99 ng/ml) and 33 (saline-treated; range of 0.50 – 17.55 ng/ml), but the increase in serum testosterone with age was delayed significantly in males treated with oxytocin (P<0.05). There was no effect of peri-adolescent oxytocin treatment on the time of first detection of sperm, or on any measures of adult male reproductive function at 36 months of age (Table 2). Detergent-resistant spermatid numbers averaged 5.7 million/g testicular parenchyma, and testicular weight was positively correlated with Sertoli cell number (R^2 = 0.77, P<0.0001).

Puberty, defined as the appearance of sperm in urine or the ejaculate of males, and regular cyclicity (3 or more luteal phases at 17–18 day intervals) or pregnancy, was tallied among individuals cumulatively by months of age for both sexes (Figure 4). Males initiated puberty months before females, from as early as 15 months of age, and all 14 males were pubertal by 22 months of age. Nine females initiated puberty at 29 months of age and all 15 were cyclic by 32 months of age (Figure 4).

Discussion

The possible effects of oxytocin treatment on reproductive function and success have not been examined previously in a non-human primate. The data reported here were collected in the context of exploring the possible effect of intra-nasal oxytocin treatment of peri-adolescent titi monkeys on their subsequent affiliative behaviors (Arias Del Razo, Berger et al. 2020). To our knowledge, this is also the first examination of the onset of puberty (as defined by sperm production in males and ovarian cyclicity in females) in titi monkeys, a species exhibiting social monogamy (Mason 1975), perhaps even sexual exclusivity (Dolotovskaya, Roos et al. 2020), forming small family units with paternal involvement in infant care (Mendoza and Mason 1986, Mendoza and Mason 1997). In males, the ratio of testicular weight to body weight was low in titi males, consistent with that reported for other monogamous primates (Clutton-Brock, Harvey et al. 1977). Our observations indicate that male titis initiate spermatogenesis several months earlier than females initiate regular cyclicity. Female ovarian cyclicity and fertility relies ultimately on oocyte release at ovulation and luteal formation. Luteal phases are either terminated at a predictable time in the absence of pregnancy or are sustained longer if pregnancy is established, all of which can be monitored in titi monkeys (Valeggia, Mendoza et al. 1999) as in other primates (Lasley, Stabenfeldt et al. 1985). Regular luteal phases with a periodicity of 17–18 days confirmed previous estimates (Valeggia, Mendoza et al. 1999) and was indicative of ovulation and the attainment of fertility or puberty as defined here. The establishment of pregnancy in some females in the present study after a single similar luteal phase further supports that view. Oxytocin had subtle effects on reproductive function in both sexes. Although oxytocin treatment had no observable effect on the onset of puberty in male titis, or their ability to establish pregnancies when paired with mature females subsequently, it temporarily delayed the puberty-associated rise in serum testosterone. In females, oxytocin-treatment increased the incidence of apparent, early ovulations around 21 months of age followed by a period of ovarian inactivity before they resumed cyclicity several months later. Apparent luteal phases in female titi monkeys as early as 26 months of age followed an extended period without elevations in PdG have been observed previously in some titi females (Valeggia 1996). Oxytocin treatment of peri-adolescents appeared to increase this otherwise infrequent event. In general therefore, it appears that, in contrast to the earlier reactivation of the hypothalamic-pituitary axis in female than male rhesus macaques (Plant 2008), gamete production in male titi monkeys is initiated long before it is in females. While not affecting the spermatogenesis, oxytocin appears capable of provoking precocious ovulation.

The sexual behavior and mating systems of primates have long held the attention of biologists in terms of discerning their potential impact on the development of physical attributes and behavior (Dixson 1983, Dixson 1998). Mating systems in particular are believed to influence the evolution of sexual dimorphisms including body weight (Plavcan and van Schaik 1997) and, at the same time, the attainment of mature body weight has long been thought to linked to the onset of puberty (Plant, Terasawa et al. 2015). With respect to body weight, males are often heavier than females, especially in species exhibiting polygyny whereas the differentials between the sexes are diminished in species exhibiting monogamy

(Clutton-Brock, Harvey et al. 1977). Even among platyrrhine species with relatively modest differences between male and female body weight, sexual conflict is believed to drive differences in the size of canine teeth (Kay, Plavcan et al. 1988). Our assessment of body weight of colony-housed titi monkeys closely align with previously published field data (Ford 1994). Females were approximately 90% of the weight of males when weight gain began to plateau in both sexes at around 30 months of age. The initiation of puberty as early as 15 months of age in male titi monkeys therefore was clearly not related to the attainment of mature body weight. Similarly, male squirrel monkeys (*Saimiri sciureus*), another platyrrhine species, experience puberty within their first year while continuing to grow well into their third (Coe, Chen et al. 1981). The apparent suppressive effect of oxytocin on the increase in body weight with age is also notable. Male rhesus macaques fed an obesity-inducing diet ate less and gained less weight when treated with oxytocin, an effect that persisted for months after treatment ceased (Blevins, Graham et al. 2015). Oxytocin appears capable of influencing multiple regulatory neural circuits, consistent with the stimulatory effect it has on glucose update in brain regions known to be associated with social behaviors (Arias Del Razo, Berger et al. 2020).

Past studies on titi monkey reproduction described cyclicity and pregnancy in females that were over 30 months in age (Valeggia, Mendoza et al. 1999) but did not specifically investigate puberty or any aspect of reproduction in males. In light of the present data, few of the females in that earlier study were likely to have been pre-pubertal. Though it was stated that conception could occur as early as 2 years of age (Valeggia, Mendoza et al. 1999), the current observations suggest that regular cyclicity (17–18 day inter-ovulatory intervals) and the ability to establish pregnancy in this colony is unlikely in females less than 2.5 years old. The closely related owl monkey (*Aotus azarae*) has a similar cycle length (≈17 days) without a clear seasonal bias and regular cyclicity is uncommon before 3 years of age in the wild (Corley, Valeggia et al. 2017). In contrast to female titi monkeys, spermatogenesis was accomplished at a much earlier age in males, though it is unknown if males were mature otherwise. Male titis exhibit agonistic displays toward unfamiliar males for instance (Cubicciotti III and Mason 1978), a response possibly associated with reproductive or more general behavioral maturity. When such male typical behaviors develop in relation to puberty is another interesting but unanswered question. Among the Primates, puberty has been examined most extensively in catarrhines (Stephens and Wallen 2013, Plant, Terasawa et al. 2015), especially in females where menses can often be detected visually. However, ovulation is not a pre-requisite for menses and, in adolescent women, menses can occur for months or even years in the apparent absence of ovulation (Vihko and Apter 1984). In contrast to the Catarrhines, the onset of puberty has not been documented in many South American species (Corley, Valeggia et al. 2017). Neotropical primates do not exhibit overt signs of menses (Mayor, Pereira et al. 2019) and the capacity to initiate puberty can be difficult to determine in species exhibiting reproductive suppression for instance, especially in those exhibiting co-operative breeding (Epple and Katz 1980, Ziegler, Savage et al. 1987, French, Bales et al. 2003). Males do not appear to be so affected (Ginther, Carlson et al. 2002). Seasonality and body weight can influence the onset of puberty in some species, female squirrel monkeys for example (Coe, Chen et al. 1981).

Oxytocin had subtle but significant effects on both female and male reproductive function. Females treated with oxytocin experienced an earlier onset of luteal phases indicative of ovulation, though not regular cyclicity, which was established months later in some cases. Oxytocin receptors are expressed in ovarian tissues of primates and may be involved in the process of luteinization (Einspanier, Jurdzinski et al. 1997). However, luteal phases were observed months after the end of treatment and an acute effect of oxytocin therefore seems unlikely, suggesting that an organizational effect at the hypothalamic-pituitary level instead. Consistent with this possibility, a 6 day treatment of juvenile rats with an oxytocin receptor antagonist can delay the onset of vaginal opening and the first estrus through effects on glial production of prostaglandin E_2 (Parent, Rasier et al. 2008). However, the effects of oxytocin on cyclicity appear to be dependent on the timing of treatment because rats treated with oxytocin for the first 6 days of post-natal life were reported to experience a delayed vaginal opening and first estrus (Withuhn, Kramer et al. 2003). As to male reproductive function, oxytocin may affect testosterone secretion. Leydig cells express oxytocin receptors (Einspanier and Ivell 1997), and testicular androgen concentrations were low in mice over-expressing oxytocin as a transgene (Ang, Ungefroren et al. 1991). Similarly, testosterone secretion by rat Leydig cells was inhibited by oxytocin both *in vitro* and *in vivo* (Adashi and Hsueh 1981, Kwan and Gower 1988, Nicholson, Guldenaar et al. 1991). Despite the possibility of direct effects on Leydig cell steroidogenesis, there was no effect on serum testosterone at 13 months of age, a month after treatments began, even though an increase at 23 months of age was delayed by oxytocin, several months after treatment ceased. The observation of delayed effects, both promoting early ovulation in females and delaying serum testosterone secretion in males months after treatment ceased, is consistent with the involvement of higher levels of the regulatory axis which is further supported by observed changes in behaviors still evident long after treatment ended (Arias Del Razo, Berger et al. 2020). How oxytocin treatment affected neural centers in the hypothalamus perhaps, the pre-optic area and arcuate nucleus for instance, would be of particular interest, especially if found to express oxytocin receptors at levels seen in centers associated with behaviors such as social bonding (Freeman, Walum et al. 2014). Further studies will be required to investigate proximate mechanisms in both sexes.

It is equally interesting that almost half of all first pregnancies failed among the females studied. This was not related to oxytocin treatment and loss occurred over a wide range of gestational stages from early to relatively late-term abortion. There was no obvious sign of illness among any of these females and only trace blood was evident in the perineal region of a few, that was found to be coincident with a drop in both urinary pregnane and estrogen conjugate concentrations. Cyclicity resumed soon after the loss of the pregnancy which also suggests these females were healthy at the time. Abbott & Hearn (Abbott and Hearn 1978) noted a high incidence (45%) of spontaneous loss of first pregnancies established in recently pubertal marmosets (*Callitrix jacchus*) but no details were provided on when losses occurred during gestation. This is remarkably similar to the rate of loss observed here in pubertal titi monkeys, suggesting that social influences are perhaps not a significant contributing factor in either case. Moreover, the wide range in the timing of loss (from as early as 33 to as late as 117 days of gestation) seen here also argues against a single or common mechanism. In any case, the rate of pregnancy loss is similar to rates seen in livestock (First and Eyestone

1988). Actual rates of loss are difficult to estimate at any stage of embryonic or fetal development (Jarvis 2020), in part because it requires constant monitoring, but these losses are thought to be highest in earlier stages in general (Chen, Di et al. 2020). Maternal age is clearly not a factor here, though fertility declines as pregnancy failures increase with age in human (Cohain, Buxbaum et al. 2017) and non-human primate (Gagliardi, Liukkonen et al. 2007) populations. What impacts the maintenance of pregnancy and ultimate fertility in pubertal females certainly warrants additional investigation.

Funding:

Grants HD071998 to KLB, SPM, TB & AJC, and OD011107 to Prasant Mohapatra/CNPRC

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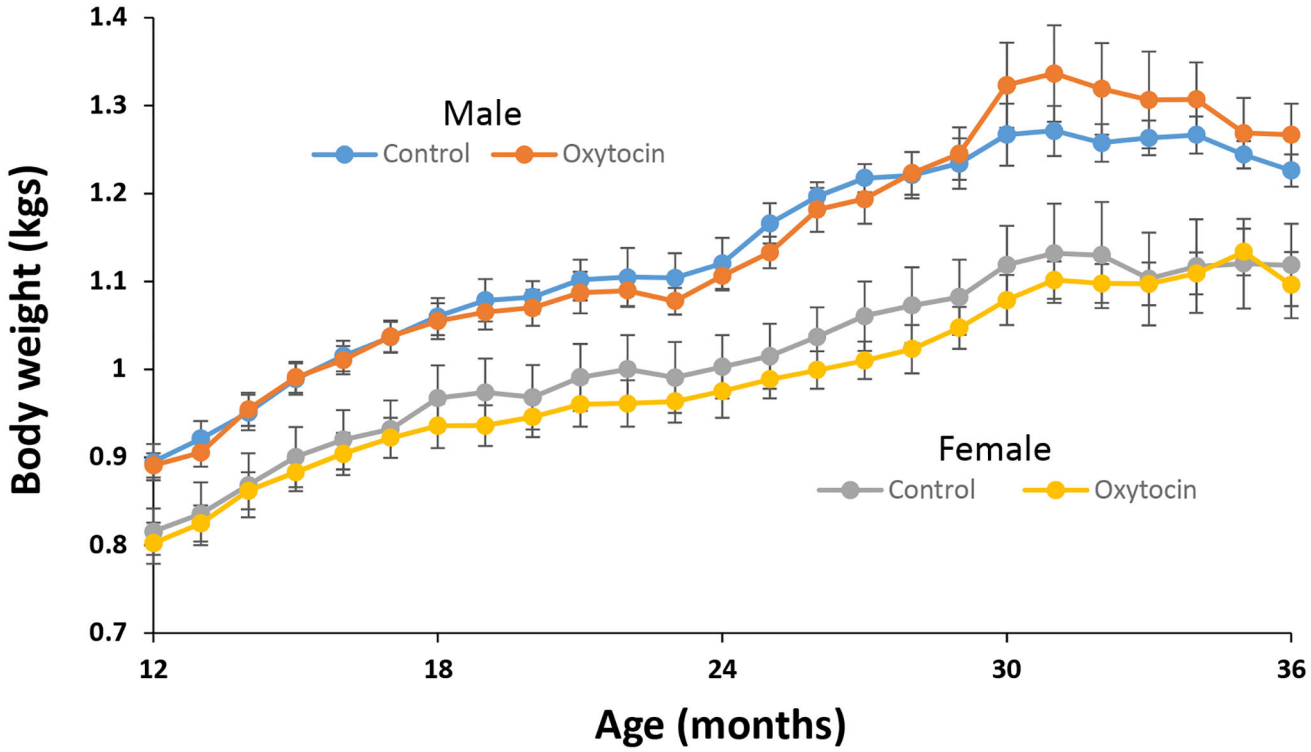
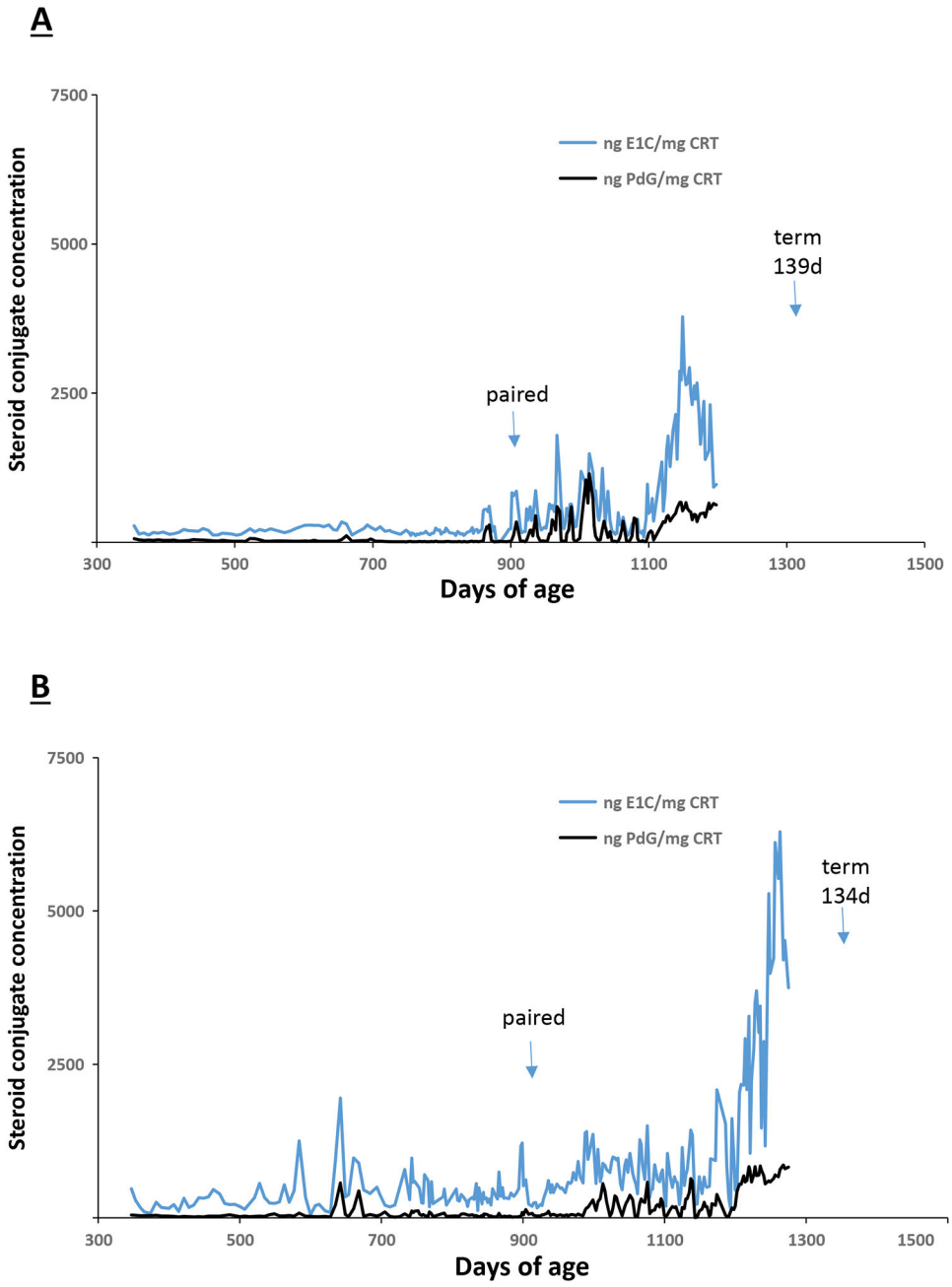


Figure 1.

Body weights in male and female titi monkeys treated with intra-nasal saline or oxytocin from 12 to 18 months of age and monitored until pregnancies were established. Shown are the means ± standard errors of the means for control (saline) and oxytocin-treated males and females.



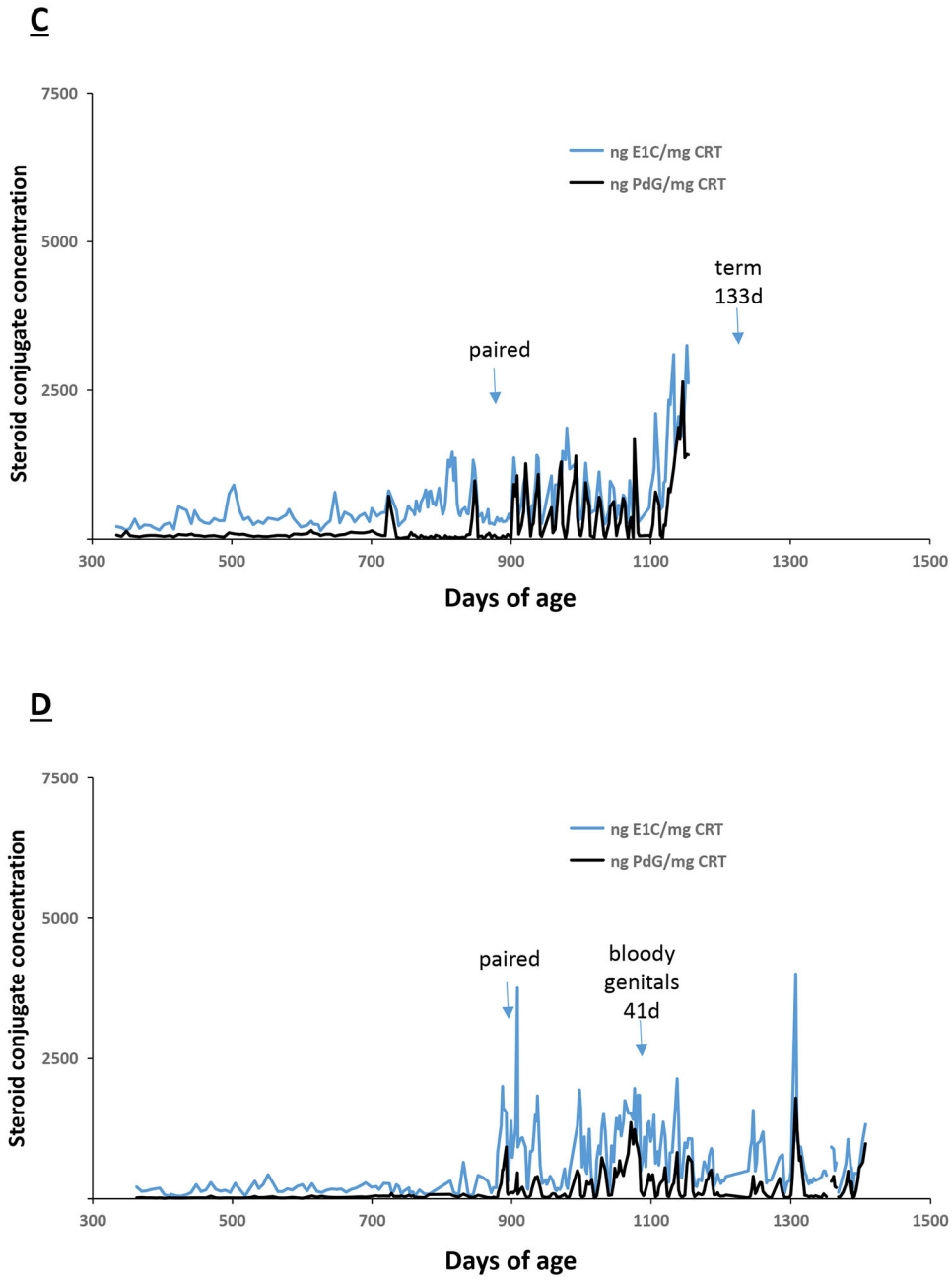


Figure 2.

Urinary pregnane (PdG) and estrogen conjugate (E1C) profiles of peri-adolescent female titi monkeys sampled three times per week from 12 months of age through to the establishment of their first pregnancy. The timing of removal from their family unit and pairing with a male are indicated, along with the birth or the loss of pregnancy as indicated by observed bloody genitals and disappearance of PdG and E1C from their urine. **A**. Cyclicity in an oxytocin-treated female experiencing 2 ovulations (860 and 902 days) before pairing at 914 days of age. Pregnancy was established and carried to term. Gestation length was 139 days. **B**. An oxytocin-treated female exhibiting two consecutive “early” ovulations from 625–700 days of age initiated regular cyclicity (984 days) after pairing (904 days). Pregnancy was

established at 1190 days after several cycles and was carried to term (134 days). **C**. A saline-treated female exhibiting “early” ovulation before initiating regular cyclicity (897 days) after pairing (893 days). A pregnancy was established (1110 days) after several cycles and was carried to term (133 days). **D**. Cyclicity was initiated (880 days) before pairing (893 days) in an oxytocin-treated female who established a pregnancy (1043 days) that failed soon after (41 days) as indicated by observed bloody genitals coincident with disappearance of PdG and E1C in urine. Regular cyclicity resumed immediately after and another pregnancy was established at 1389 days which went to term successfully.

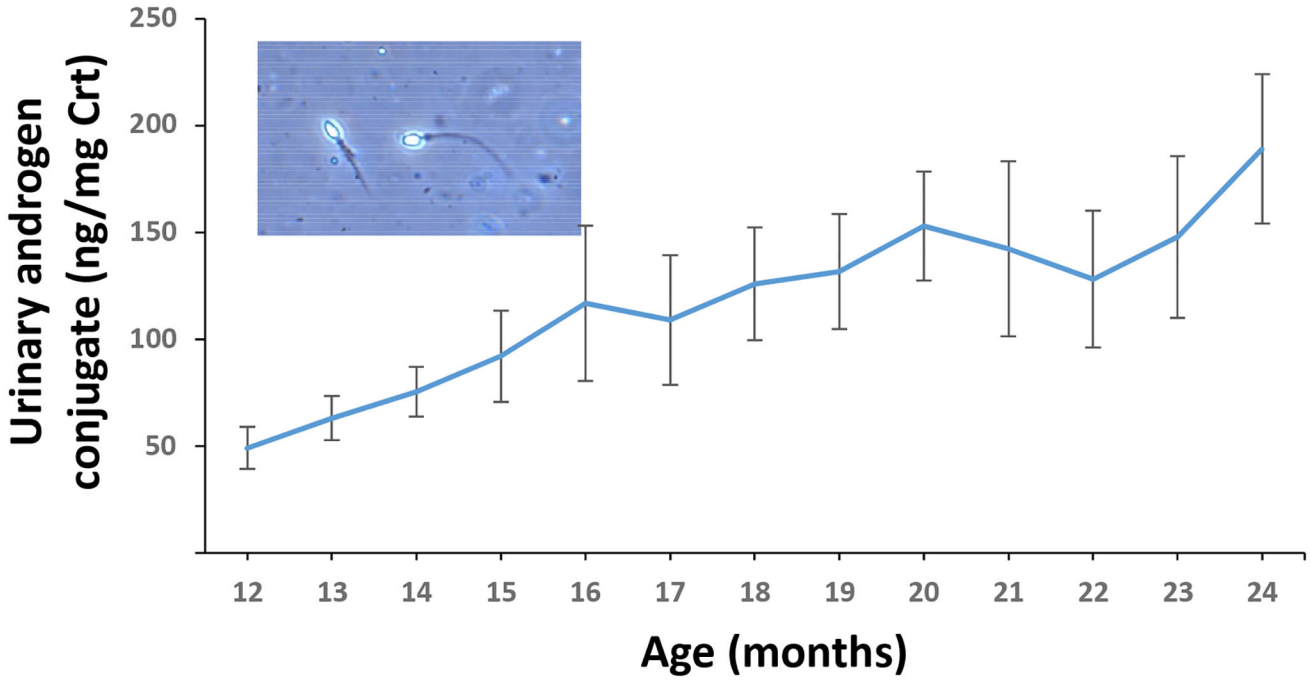


Figure 3.

Urinary immuno-reactive androgen concentrations (ng/mg Crt) in males from 12 to 24 months of age. Sperm (inset) was detected in the urine of all males by 22 months of age, confirming that all had initiated spermatogenesis.

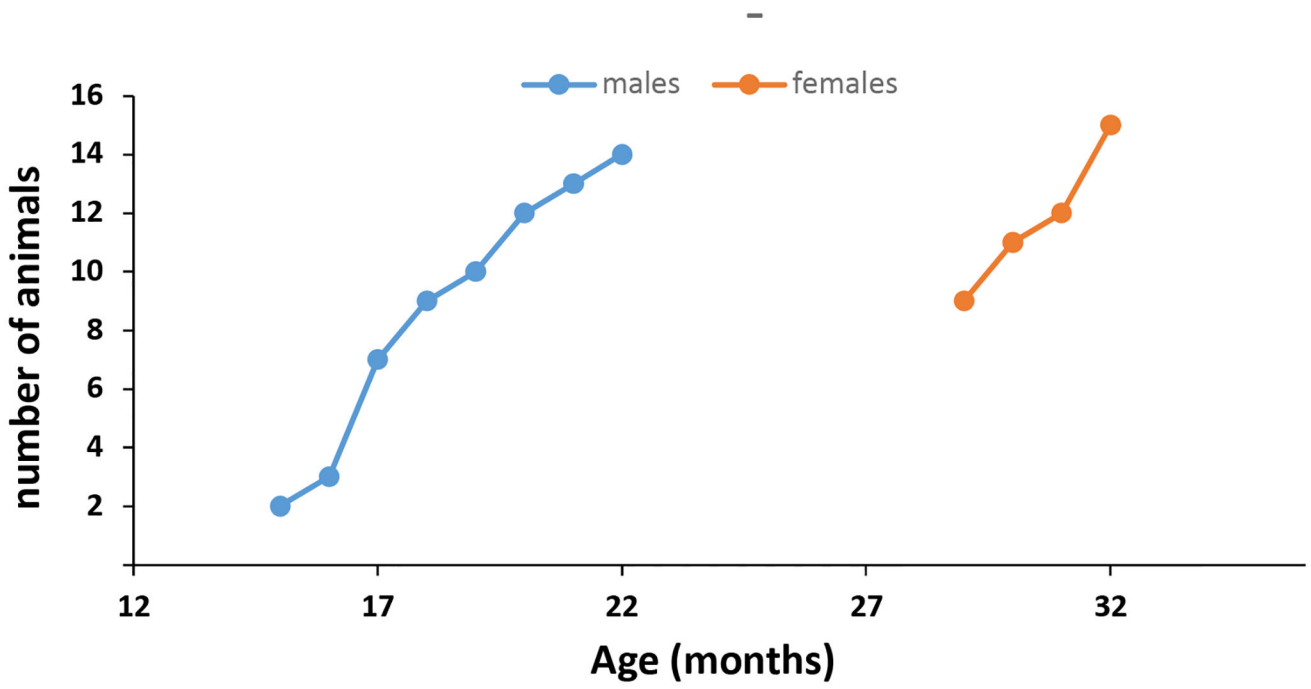


Figure 4.

Cumulative tally (number of animals, y-axis) of the onset of puberty in male and female titi monkeys from 12 – 32 months of age based on the appearance of sperm in voided urine or stimulated ejaculates (males) or the onset of regular cyclicity (females) based on urinary pregnane and estrogen conjugate profiles.

Table 1

Serum testosterone concentration (ng/ml) in male titi monkeys treated with saline or oxytocin by intra-nasal spray. Blood was drawn opportunistically under anesthesia that was induced before subjects underwent PET imaging of the cranium as previously reported (Arias Del Razo, Berger et al. 2020). Shown are back transformed log means and the standard errors of the means. Comparisons between log-transformed means with different superscripts differ significantly, *P < 0.05.

	Age (weeks)		
Treatment	**13**	**23**	**33**
Saline	0.40 ± 0.70	1.99 ± 0.81[a]	2.29 ± 1.46
Oxytocin	0.66 ± 1.41	0.76 ± 0.87[b]	2.01 ± 1.01

*P < 0.05

Table 2.

Testicular characteristics in titi monkeys at 36 month of age after daily intra-nasal treatment with saline or oxytocin from 12–18 months of age.

	Saline	Oxytocin
Testis weight (g)	0.27 ± 0.03	0.23 ± 0.03
Sertoli cells (millions)/ testis	22.7 ± 2.6	19.7 ± 2.8
Spermatogonia (UTF1-positive cells) millions/testis	7.01 ± 1.25	3.98 ± 1.28
Age at conception (days)	937 ± 12	932 ± 12
Oxytocin receptor expression (Δ Ct)	2.87 ± 0.4	3.38 ± 0.43
Sperm/ejaculate at 34 to 36 months ($\times 10^5$)	1.54 ± 1.34	5.08 ± 1.34