Variation in Circulating and Excreted Estradiol Associated With Testicular Activity in Male Marmosets

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Concentrations of estradiol (E_2) are high in the urine of male marmosets, and links between E_2 and paternal behavior have been proposed in black tufted-ear marmosets, *Callithrix kuhlii*. However, it is not clear whether urinary E_2 in male marmosets: 1) represents production of E_2 associated with testicular activity, 2) is associated with adrenal steroid production, or 3) merely reflects peripheral conversion of T to E_2 prior to excretion. We tested the hypothesis that urinary E_2 in male marmosets represents estrogen production-associated activity in the hypothalamus-pituitary-gonad (HPG) axis. We treated adult male marmosets with gonadotropin-releasing hormone (GnRH), and used saline-treated males as controls. We collected blood and urine samples from males before and after treatment, and assayed them for testosterone (T), estradiol (E_2), and cortisol (CORT). Treatment with GnRH increased circulating T and E_2, and prevented decreases in levels of urinary T and E_2. Moreover, changes in plasma and urinary E_2 after treatment were positively correlated with post-treatment changes in T. Thus, our data are consistent with both plasma and urinary E_2 in male marmosets increasing as a result of testicular stimulation. However, treatment with GnRH did not affect plasma or urinary CORT concentrations of males, suggesting that the E_2 excreted by males is not of adrenal origin. We also compared urinary T, E_2, and CORT levels between intact and castrated male common marmosets (*Callithrix jacchus*). Urinary concentrations of T and E_2, but not CORT, were significantly lower in castrated than in intact males, further suggesting that E_2 in male marmosets varies with testicular activity. Am. J. Primatol. 56:27–42, 2002.   © 2002 Wiley-Liss, Inc.

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INTRODUCTION

Androgens are associated with reproduction in male mammals, and play an important role in mediating features of reproductive physiology and behavior [Eleftheriou & Sprott, 1975; French et al., 1975; Bronson, 1989]. Among primates,

several lines of evidence raise the possibility that estrogens are also important in mediating masculine physiology and behavior. Conversion of testosterone (T) to estradiol (E_2) via the enzyme aromatase in the central nervous system is an important regulator of sexual behavior in male macaques [e.g., Zumpe et al., 1993; Zumpe & Michael, 1994]. In addition, high concentrations of circulating estrogens in males have been measured during early postnatal life and puberty in cynomolgus monkeys (*Macaca fascicularis* [Meusy-Dessolle & Dang, 1985]), and high estrogen levels in males have been observed throughout life in owl monkeys (*Aotus trivirgatus* [Setchell & Bonney, 1981]). Moreover, estrogen levels are lower in castrated than intact common marmosets (*Callithrix jacchus* [Lunn, 1978]), and urinary concentrations of estrogens increase in adult male cotton-top tamarins (*Saguinus oedipus*) when they are exposed to adult females [Brand, 1987], indicating a possible link between estrogen and reproductive function in these species.

Along these lines, in prior work we observed associations between T, E_2, and infant-carrying behavior in male black tufted-ear marmosets (*Callithrix kuhlii*) suggesting that these hormones may be incompatible with paternal behavior in *C. kuhlii*. In particular, urinary T and E_2 levels declined in male marmosets after their young were born, and reached their lowest levels during the period in which males carried infants at peak rates [Nunes et al., 2000]. Moreover, both urinary T and E_2 levels were lower in males who carried infants at high compared to low rates, and urinary T and E_2 levels were correlated with each other during the overall period in which males carried infants [Nunes et al., 2001]. Urinary E_2 in male *C. kuhlii* may represent estrogen production associated with activity along the hypothalamus-pituitary-gonad (HPG) axis. This would support the notion that E_2 and paternal behavior are linked to each other in male *C. kuhlii*. Metabolic clearance studies using radiolabeled steroids indicate that 20–30% of circulating E_2 in male humans and cynomolgus monkeys is in fact derived from direct testicular synthesis [MacDonald et al., 1979; Bourget et al., 1988; Greenspan and Strewler, 1997]. Alternatively, urinary E_2 in male *C. kuhlii* may merely reflect the conversion of T to E_2 by aromatase in central or peripheral tissue. According to this scenario, E_2 would have no direct relationship to paternal behavior in male *C. kuhlii*.

A recent study on cotton-top tamarins suggested that E_2 excretion in males was associated with variation in gonadal function [Ziegler et al., 2000]. Inhibition of the HPG axis by a gonadotropin-releasing hormone antagonist (Antide) led to significantly reduced excreted T and E_2 in male tamarins. Here we adopt a complementary strategy to Ziegler et al. [2000] for testing the hypothesis that E_2 in male *C. kuhlii* is produced and released in response to activity along the HPG axis. We stimulated the HPG axis with a GnRH challenge, which stimulates the pituitary to release gonadotropins, which in turn promote steroidogenesis and steroid secretion by the testes. Following GnRH stimulation, concentrations of pituitary gonadotropins typically peak about 30 min after injection and attenuate to near baseline levels approximately 90 min after treatment [Abbott, 1993]. We injected experimental subjects with GnRH, and used saline-treated males as controls. Blood and urine samples were collected from males before and after treatment, and later assayed for T and E_2. To assess potentially confounding effects of activity along the hypothalamus-pituitary-adrenal (HPA) axis on E_2 levels, we also assayed samples for cortisol (CORT). In male marmosets, E_2 is the predominant urinary estrogen [e.g., Lunn, 1978; Fite and French, 2000; Nunes et al., 2000, 2001], and CORT is the main adrenal glucocorticoid [Smith & French, 1997]. We reasoned that if E_2 levels vary with HPG activity in male marmosets,

then concentrations of plasma and urinary T and E_2 should be higher following injection in GnRH-treated males than in saline-treated males.

To further evaluate sources of variation in urinary E_2 among male marmosets, we compared urinary concentrations of T, E_2, and CORT between intact and castrated males. We were unable to use black tufted-ear marmosets in this experiment, so we used common marmosets (*C. jacchus*), which are very similar to black tufted-ear marmosets with respect to behavior, ecology, and evolutionary history [Nowak, 1991]. In fact, earlier taxonomies of callitrichid primates placed *C. kuhlii* as a subspecies of *C. jacchus* (= *C.j. penicillata* [Hershkovitz, 1977]). We predicted that if urinary concentrations of E_2 fluctuate with testicular but not adrenocortical activity in male common marmosets, then levels of urinary T and E_2, but not CORT, should be lower among castrated than among intact males. We predicted that if urinary E_2 varies with adrenocortical but not testicular activity, then levels of urinary T, but not E_2 or CORT, should be lower among castrated than among intact males.

METHODS
Subject Animals

Black tufted-ear marmosets are small, tree-dwelling primates native to forests near the Atlantic coast of South America [Nowak, 1991]. Like other members of the family Callitrichidae (tamarins and marmosets), black tufted-ear marmosets are cooperative breeders, living in groups typically composed of a breeding male and female who form a long-term social bond with each other, and their offspring [Schaffner et al., 1995]. Males reach reproductive maturity at about 12 mo of age [French & Schaffner, 1995], and females at about 12–15 mo [Smith et al., 1997]. We included in our study 12 reproductively-mature male marmosets housed at the Callitrichid Research Center at the University of Nebraska–Omaha. Animals ranged in age from 43–125 mo (mean age = 70.8 ± 24.0 (SD) mo).

Marmosets were kept in large indoor enclosures ($1.6 \times 0.9 \times 2.4$ or $1.2 \times 0.9 \times 2.4$ m), each equipped with a plastic sleeping tube, and platforms and natural branches for climbing. One male was being housed temporarily by himself pending pairing with a mate, two males comprised a father–son pair residing together, four males each lived with a female pairmate, and five males each were housed with a female mate and young. Visibility between enclosures containing different groups in the same room of the research center was restricted by large sheets of opaque corrugated plastic. Animals were fed once daily at approximately 0800 hr, and water was provided ad libitum. Specific details of animal care have been described by Schaffner et al. [1995].

GnRH Treatment, and Collection of Blood and Urine Samples

Male *C. kuhlii* in the experiment were assigned randomly to either an experimental (GnRH treatment, n = 8) or control (saline treatment, n = 4) group. We collected first-void urine samples from marmosets using noninvasive, stress-free techniques [French et al., 1996] between 0600–0800 hr. Specifically, animals urinated into small, hand-held pans in return for food rewards. Immediately after collecting urine from each marmoset, we lured it into a single-animal transport cage ($30 \times 32 \times 34$ cm) using a food incentive. Cages were placed in a quiet holding room in the research center, and food and water were available to animals ad libitum throughout the day.

Between 0845–1000 hr, we collected blood samples (0.1 ml) by femoral venipuncture. Immediately after sample collection, we administered an intravenous injection of either 2 µg of GnRH (Sigma Chemical, St. Louis, MO) in 100 µl of sterile saline, or 100 µl of saline alone. For these procedures, males were moved one at a time for a short period to a quiet workroom in the research center, where they were restrained briefly by hand. We collected a second blood sample 88–104 min after injection (mean = 94.3 ± 7.4 (SD) min). After the second blood sample was collected, the small cages in which the animals were held were placed on clean, stainless steel urine collection pans in the quiet holding room. We checked trays at 1-hr intervals until 1600 hr, and collected urine when it was present. Animals were returned to their home enclosures between 1600–1630 hr. Finally, first-void urine samples were collected the morning after the experimental treatment day between 0600–0800 hr, again using noninvasive, stress-free techniques.

Blood samples were centrifuged at 2,000 g for 20 min, and the plasma portion was saved and stored at –20°C until assayed for hormones. Urine samples were centrifuged at 700 RPM for 2 min to separate urine from sediments, and the supernatant was collected and stored at –20°C until assayed. We note that one male (Coimbra) did not urinate for >25 hr after being treated with GnRH, and so was excluded from our analysis of urinary hormones.

Urinary Steroids in Castrate Common Marmosets

To further assess sources of variation in urinary E_2 levels of male marmosets, we compared urinary steroid concentrations between intact and castrated animals. Black tufted-ear marmosets were not available for this experiment, so we used common marmosets, which are closely related to black tufted-ear marmosets. Urine samples were collected from intact males housed at Kent State University, and from castrated males housed at the University of Wisconsin, Madison. Husbandry of these animals has been described by Tardif and Bales [1997] and Saltzman et al. [1994]. First-void urine samples were collected in the morning, stored at –20°C, and shipped to the University of Nebraska–Omaha where they were assayed for T, E_2, and CORT using techniques described below.

Hormone Assays

We measured plasma concentrations of T, E_2, and CORT with ^{125}I radioimmunoassay kits (ICN Diagnostics, Costa Mesa, CA). Each kit was validated for use with marmosets by demonstrating parallelism between serial dilutions of marmoset plasma and the standards generated with kit calibrators. Intra-assay coefficients of variation for T, E_2, and CORT, estimated from replicate determinations of samples, were 2.6%, 2.5%, and 2.9%, respectively. Interassay coefficients of variation, based on repeated measurement of a pool of plasma across two assays, were 15.2% for T, 16.6% for E_2, and 11.4% for CORT. Minimum assay sensitivity was 0.2 ng/ml for T, 10 pg/ml for E_2, and 1.0 µg/dl for CORT.

We measured urinary concentrations of T, E_2, and CORT with enzyme immunoassays developed and validated for use with *C. kuhlii* [Smith & French, 1997; Fite & French, 2000; Nunes et al., 2000]. Urinary T and E_2 concentrations were measured in hydrolyzed (20 µl β-glucuronidase, G-0876; Sigma Chemical Co., St. Louis, MO) and diethyl ether-extracted urine samples [Nunes et al., 2000]. Recovery of hormones during extractions, determined by assessing recovery of ^{3}H-T and ^{3}H-E_2 in spiked samples, was 91.6% for T and 75.8% for E_2. We adjusted hormone levels obtained from assays to correct for losses during extrac-

tions. Urinary free CORT was measured in unhydrolyzed and unextracted urine samples diluted in assay buffer. Hormone concentrations in urine samples were evaluated in a total of four T assays, four E_2 assays, and three CORT assays. The precision of T and E_2 assays was determined by hydrolyzing, extracting, and assaying in duplicate samples from two pools of male marmoset urine, containing either high or low concentrations of T and E_2. Similarly, the precision of CORT assays was estimated by assaying in duplicate samples from pools of marmoset urine containing high or low concentrations of CORT. Intra-assay coefficients of variation for high and low concentration pools, respectively, were 4.5% and 4.5% for T, 5.0% and 1.9% for E_2, and 6.6% and 3.1% for CORT. Interassay coefficients of variation for the same high and low concentration pools, respectively, were 16.8% and 9.2% for T, 12.3% and 9.1% for E_2, and 18.8% and 14.0% for CORT. To minimize the impact of interassay variation on analysis of individual patterns of response to GnRH or saline treatment, all samples from specific individuals were evaluated for steroids in the same assay run.

To control for variation in solute concentrations of urine samples, we expressed the mass of T, E_2, and CORT determined by our assays per mg of creatinine, measured using a modified Jaffe end-point assay [Tietz, 1976], previously described and validated for black tufted-ear marmosets [French et al., 1996].

Statistics

Dependent measures monitored included plasma and urinary concentrations of T, E_2, and CORT, and absolute and proportional changes in these hormones after treatment with GnRH or saline. We evaluated our data using mixed-design analysis of variance (ANOVA) [Sokal and Rohlf, 1981], with time as a repeated, within-subjects factor, and treatment status as a between-subjects factor. We compared hormone levels between consecutive time intervals with paired t-tests. We compared changes in hormone levels between GnRH- and saline-treated males using t-tests for independent samples, corrected for nonhomogeneous variances when necessary, and nonparametric tests when data did not follow the normal distribution. Significance levels of multiple pairwise comparisons were adjusted with Holm's procedure [Neter et al., 1996]. We used correlation analysis to evaluate the relationship between plasma vs. urinary concentrations of hormones, and plasma vs. urinary changes in hormones following our manipulations. We also used correlation analysis to evaluate concentrations of E_2 vs. concentrations of T or CORT in plasma, and in urine, and to evaluate postmanipulation changes in E_2 vs. changes in T or CORT in plasma, and in urine. Urinary hormone concentrations of intact and castrated male common marmosets were compared with t-tests for independent samples. Mean values are presented ± one standard error of the mean unless otherwise indicated. We defined observed differences to be significant when $P \leq 0.05$.

RESULTS
Plasma Hormones

Treatment with GnRH influenced both T and E_2 secretion in male *C. kuhlii*. Our analysis of plasma T revealed a significant interaction between time and treatment status (Fig. 1a) (ANOVA, $F_{1,10} = 5.868$, $P = 0.036$), suggesting a differential response to treatment between GnRH- and saline-injected males. We similarly observed an interaction between time and treatment status that approached significance in our analysis of E_2 (Fig. 1b) (ANOVA, $F_{1,10} = 4.462$, $P = 0.061$). In
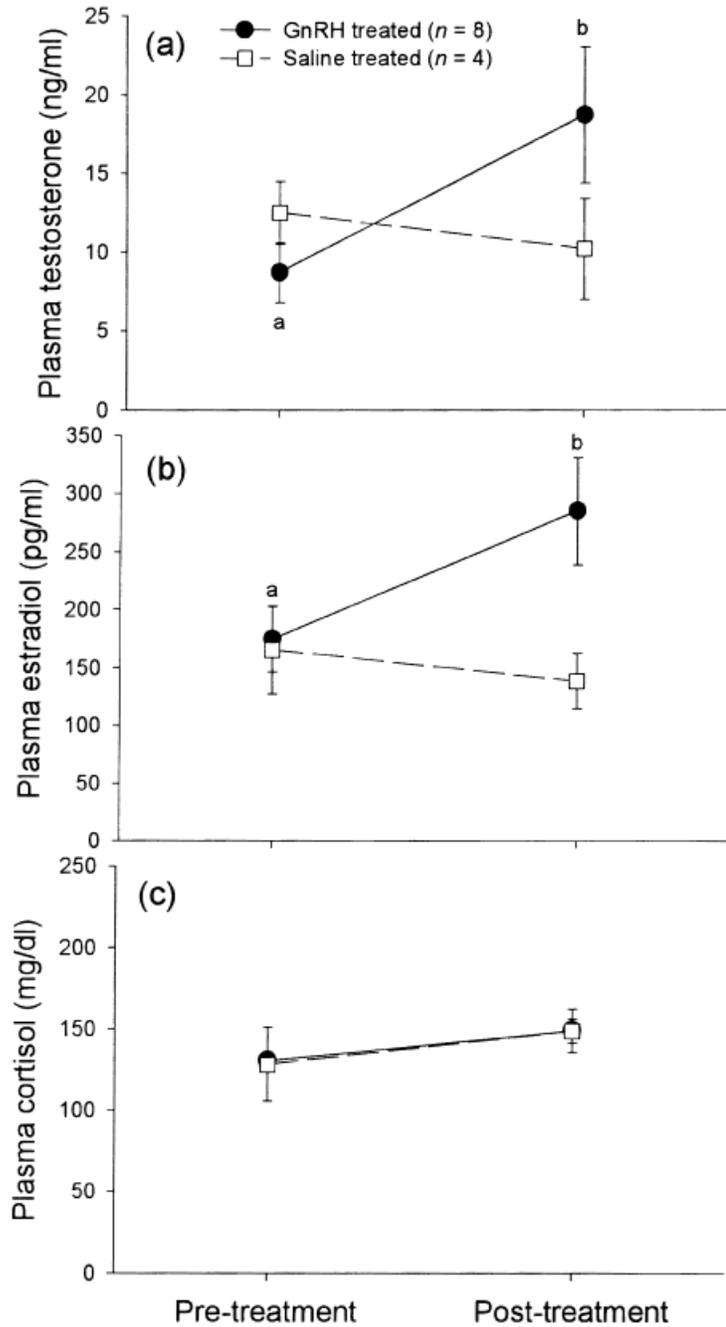
Fig. 1. Plasma concentrations of (**a**) testosterone, (**b**) estradiol, and (**c**) cortisol of male *C. kuhlii* before and after treatment with GnRH or saline. Different lowercase letters indicate significant differences between pre- and post-treatment hormone levels in GnRH-injected males.

particular, plasma T and E_2 concentrations increased significantly after injection in GnRH- but not saline-treated males (Fig. 1a and b). The effect of GnRH treatment on plasma CORT levels differed from its effect on levels of plasma T and E_2. We did not observe an interaction between time and treatment status in our evaluation of plasma CORT, and plasma CORT concentrations of males did not increase after injection with either GnRH or saline (Fig. 1c).

Analysis of proportional changes in plasma hormone concentrations after injections with GnRH or saline revealed a similar effect of GnRH treatment: T and E_2 showed significant proportionate increases over baseline in GnRH- but not saline-treated males (Fig 2a) (Kruskal-Wallis test, T: $H_1 = 32.0$, $P = 0.007$, E_2: $H_1 = 29.0$, $P = 0.027$). By contrast, proportional changes in plasma CORT concentrations were small and did not differ between GnRH- and saline-treated males (Fig. 2a). Thus, our results indicate overall that GnRH treatment has similar influences on plasma T and E_2 concentrations in male *C. kuhlii*, but has no evident effect on plasma concentrations of CORT. We also note that neither absolute levels of nor proportional changes in any of the plasma hormones we examined varied significantly with the conditions in which males were housed.

Urinary Hormones

Absolute concentrations of urinary T, E_2, and CORT varied significantly over time in male *C. kuhlii* (Tables I–III) (ANOVA, T: $F_{2,18} = 5.049$, $P = 0.018$, E_2: $F_{2,18} = 5.049$, $P = 0.018$, CORT: $F_{2,18} = 8.831$, $P = 0.002$). However, we observed no significant overall differences between GnRH- and saline-treated males in absolute concentrations of any of these hormones, and no significant interactions between treatment status and time.

Analysis of proportional changes in urinary hormone concentrations on the day of the experiment after GnRH or saline treatment, however, revealed an effect of GnRH on levels of T and E_2. Urinary concentrations of T and E_2 tended to decline in male *C. kuhlii* following injections with GnRH or saline, but the declines were significantly smaller in GnRH- than in saline-treated males (Fig. 2b) (Kruskal-Wallis test, T: $H_1 = 25.0$, $P = 0.038$, E_2: $H_1 = 25.0$, $P = 0.038$). By contrast, urinary CORT concentrations tended to increase after animals were injected, but there was no significant difference between GnRH- and saline-treated animals in these increases (Fig. 2b). Thus, our analysis here indicates that GnRH treatment prevented decreases in urinary T and E_2 concentrations, but had no evident effect on urinary CORT levels. We also note that neither absolute levels of nor proportional changes in any of the urinary hormones we examined varied significantly with the conditions in which males were housed.

Correlations Among Hormone Secretion and Excretion Profiles

Proportional changes in plasma concentrations of T, E_2, or CORT following GnRH or saline treatment were not significantly correlated with proportional changes in urinary levels of these hormones. Thus, we observed no associations between plasma and urinary hormonal changes in response to our manipulations.

We also evaluated whether correlations existed between plasma and urinary E_2 levels, and plasma and urinary levels of T and CORT. Concentrations of E_2 were significantly correlated with concentrations of T in urine (n = 66, $r = 0.465$, $P < 0.001$), but not plasma. Furthermore, proportional changes in E_2 levels following injection with GnRH or saline were correlated with proportional changes in T in both plasma (Fig. 3a) (n = 12, $r = 0.709$, $P < 0.001$) and urine (Fig. 3b)
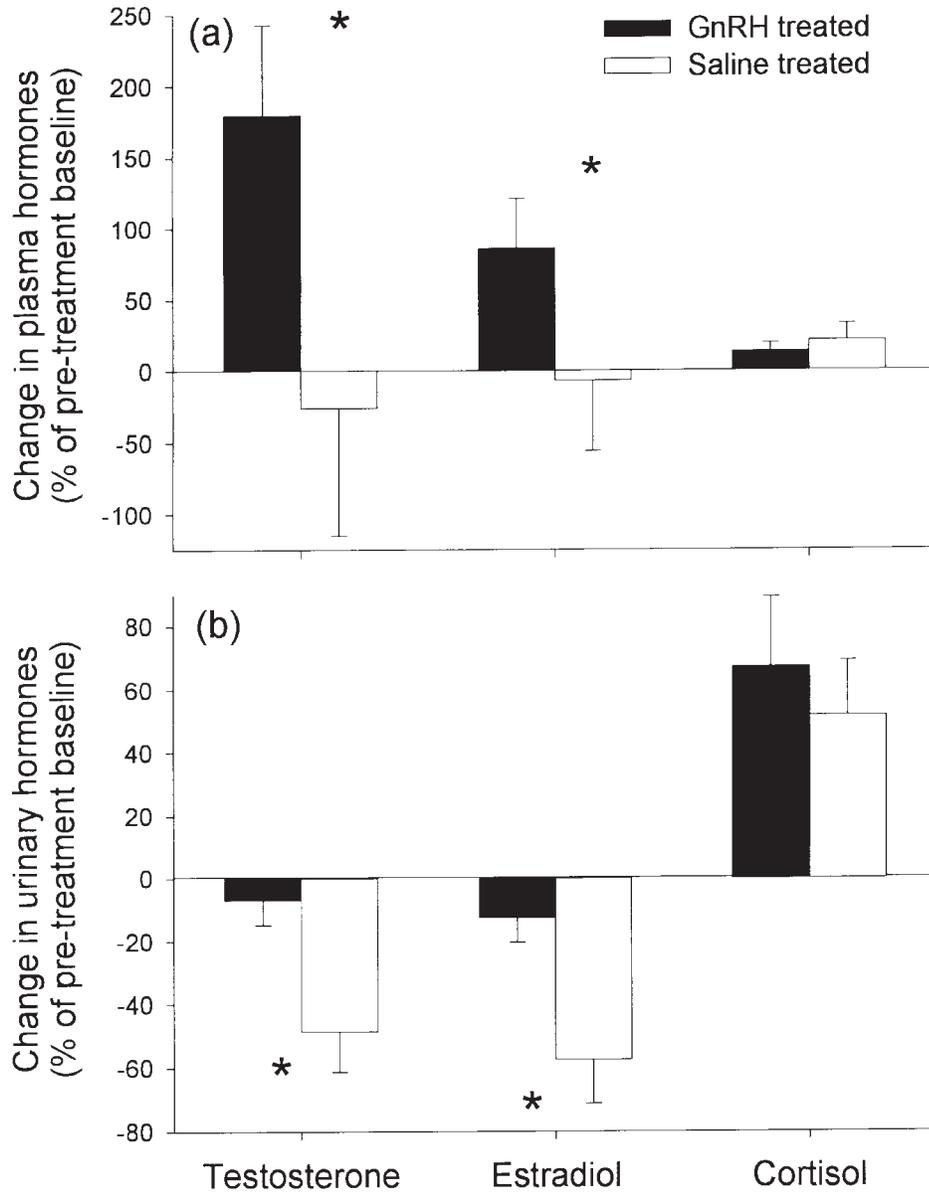
Fig. 2. Proportional changes in testosterone, estradiol, and cortisol in the (**a**) plasma and (**b**) urine of male *C. kuhlii* after treatment with GnRH (n = 8 for plasma hormones, n = 7 for urinary hormones) or saline (n = 4). Data depict changes during the ~7-hr period following treatment. Asterisks indicate significant differences between GnRH- and saline-treated males.

TABLE I. Changes in Urinary Testosterone Associated With GnRH Treatment in Male *C. kuhlii*

ID	Treatment group	Urinary testosterone (µg/mg creatinine)				
		Pre-treatment		Post-treatment		Next day
Cal	GnRH	3.3		2.6		0.7
Caly	GnRH	3.3		3.4		2.4
Coi	GnRH	N/A		N/A		N/A
Fig	GnRH	2.2		1.7		1.2
Hob	GnRH	3.5		4.5		1.6
Igg	GnRH	1.9		1.6		1.4
Ken	GnRH	4.9		3.4		1.0
Mow	GnRH	2.4		2.7		2.6
Ell	Saline	5.2		3.1		4.6
Hea	Saline	6.5		5.8		3.0
Kip	Saline	2.6		0.8		2.2
Ren	Saline	4.2		1.2		3.3
Mean	GnRH	3.1 ± 0.4		2.8 ± 0.4	>*	1.6 ± 0.3
Mean	Saline	4.6 ± 0.8	>*	2.7 ± 1.1		3.3 ± 0.5
Mean	Overall	3.6 ± 0.4	>*	2.8 ± 0.4		2.2 ± 0.4

*Paired *t*-test, $P \leq 0.05$.

(n = 11, $r = 0.785$, $P < 0.001$). By contrast, we observed no significant correlations between E_2 and CORT levels in plasma or urine, with respect to either absolute levels, or proportional changes after GnRH or saline treatment (Fig. 3c and d). Thus, we observed significant associations between E_2 and T levels but not between E_2 and CORT levels.

   When we evaluated urinary hormone concentrations of male common marmosets, we observed that removal of the gonads affects both urinary T and E_2

TABLE II. Changes in Urinary Estradiol Associated With GnRH Treatment in Male *C. kuhlii*

ID	Treatment group	Urinary estradiol (µg/mg creatinine)				
		Pre-treatment		Post-treatment		Next day
Cal	GnRH	5.1		5.2		2.0
Caly	GnRH	10.2		7.2		8.1
Coi	GnRH	N/A		N/A		N/A
Fig	GnRH	1.9		1.4		1.3
Hob	GnRH	6.1		8.5		4.0
Iggy	GnRH	3.5		3.2		2.5
Ken	GnRH	10.2		4.3		2.1
Mow	GnRH	6.2		6.1		2.8
Ell	Saline	2.9		1.9		2.5
Hea	Saline	6.3		5.1		6.1
Kip	Saline	6.8		2.0		9.7
Ren	Saline	21.9		3.1		4.5
Mean	GnRH	6.2 ± 1.2		5.1 ± 0.9	>*	3.2 ± 0.9
Mean	Saline	9.5 ± 4.2		3.0 ± 0.7		5.7 ± 1.5
Mean	Overall	7.4 ± 1.7		4.4 ± 0.7		4.1 ± 0.8

*Paired *t*-test, $P \leq 0.05$.

TABLE III. Changes in Urinary Cortisol Associated With GnRH Treatment in Male *C. kuhlii*.

ID	Treatment group	Urinary cortisol (µg/mg creatinine)				
		Pre-treatment		Post-treatment		Next day
Cal	GnRH	24.0		21.6		8.9
Caly	GnRH	43.9		49.9		29.7
Coi	GnRH	N/A		N/A		N/A
Fig	GnRH	13.8		34.2		14.5
Hob	GnRH	45.2		66.0		28.3
Igg	GnRH	28.2		40.9		31.7
Ken	GnRH	14.7		29.4		24.4
Mow	GnRH	19.4		44.1		28.3
Ell	Saline	35.7		62.7		41.7
Hea	Saline	28.9		33.3		54.9
Kip	Saline	36.3		53.0		26.0
Ren	Saline	21.9		39.5		21.7
Mean	GnRH	27.0 ± 4.9	<*	40.9 ± 5.5	>*	23.7 ± 3.3
Mean	Saline	30.7 ± 3.4	<*	47.1 ± 6.6		36.1 ± 7.6
Mean	Overall	$28.4 + 3.3$	<*	$43.1 + 4.2$	>*	$28.2 + 3.7$

*Paired t-test, $P \leq 0.05$.

concentrations, but not levels of CORT excretion. Intact males had significantly greater T and E_2 levels than castrated males (Fig. 4a and b) (t-test for independent samples, T: $t_{6.2} = 4.829$, $P = 0.003$, E_2: $t_{14} = 2.244$, $P = 0.042$). By contrast, urinary CORT levels did not differ significantly between intact and castrated males (Fig. 4c) ($t_{14} = 0.845$, $P = 0.412$). Thus, testicular activity appears to influence E_2 levels in male common marmosets, as it does in male black tufted-ear marmosets.

DISCUSSION

Results of our GnRH challenge experiment support the notion that HPG activity is a source of variation in the E_2 levels of male *C. kuhlii*. Treatment of males with GnRH caused increases in plasma T and E_2, and prevented decreases in urinary T and E_2. Patterns of change in plasma and urinary E_2 among both GnRH-treated and control males in our experiment closely resembled variation in T (Figs. 1 and 2, Tables I and II), and proportional changes in plasma and urinary E_2 levels of males after treatment with GnRH or saline were significantly correlated with proportional changes in T (Fig. 3). Thus, our results are consistent with the hypothesis that E_2 plays a role in the reproductive physiology of male *C. kuhlii*. The findings contribute to the notion that circulating and excreted estrogens in male callitrichids are derived from activity in the testes [Ziegler et al., 2000] and also contribute to the growing body of data suggesting that estrogens play a role in regulating behavior, physiology, and reproduction in male primates [e.g., Lunn, 1978; Setchell & Bonney, 1981; Meusy-Dessolle & Dang, 1985; Brand, 1987; Zumpe et al., 1993; Zumpe & Michael, 1994]. Nunes et al. [2000, 2001] specifically proposed an association between E_2 and paternal behavior in *C. kuhlii* in which E_2 interferes with the expression of infant-carrying behavior, possibly by promoting sexual behavior.
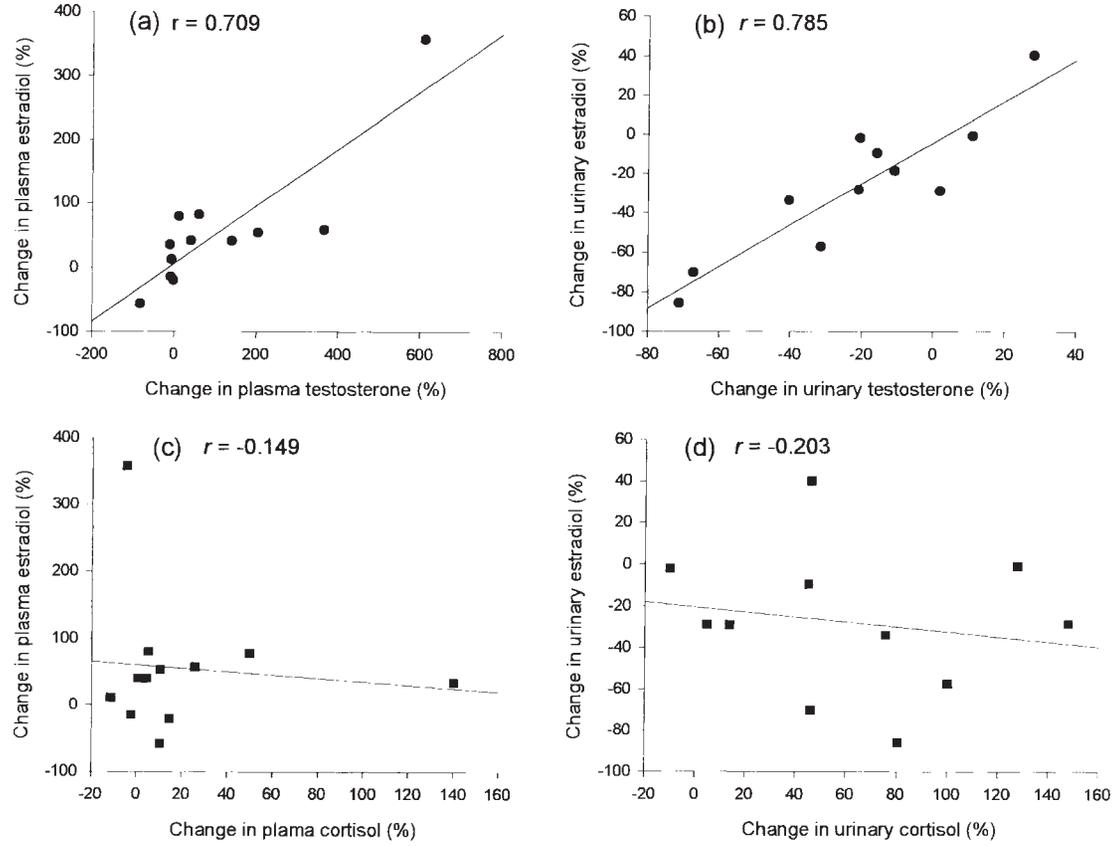
Fig. 3. Scatter plots depicting proportional changes in (**a**) plasma and (**b**) urinary estradiol vs. testosterone, and (**c**) plasma and (**d**) urinary estradiol vs. cortisol following treatment of male *C. kuhlii* with GnRH or saline.
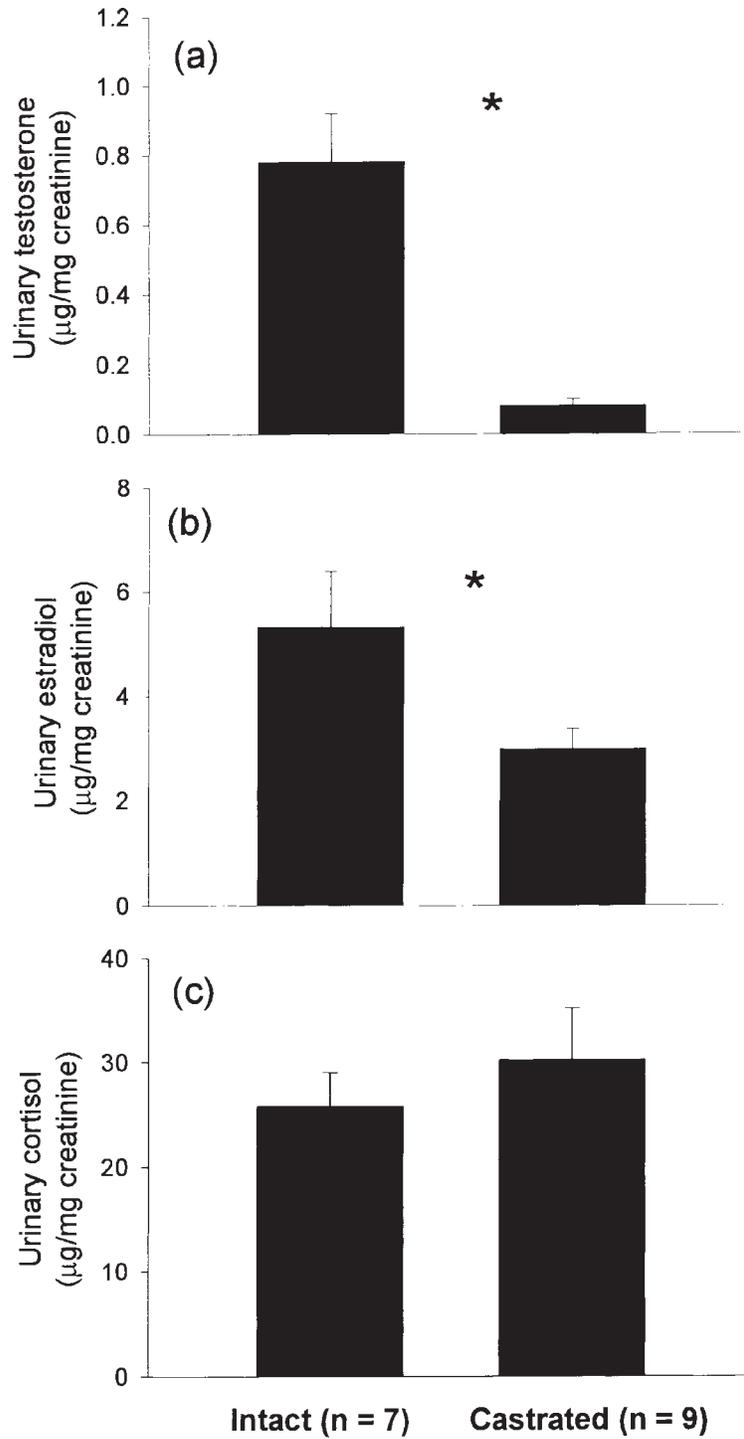
Fig. 4. Urinary concentrations of (**a**) testosterone, (**b**) estradiol, and (**c**) cortisol in male *C. jacchus*. Asterisks indicate significant differences between intact and castrated males. Sample sizes are indicated on the graph.

Concentrations of urinary T and E_2 decreased on the day following GnRH or saline treatment among GnRH- but not saline-treated males in our study. These declines in T and E_2 on the day after GnRH treatment are consistent with negative feedback of GnRH-induced increases in circulating gonadal hormones on the hypothalamus and pituitary resulting in decreased release of gonadotropins and reduced testicular activity [see Pieper et al., 1995; Bittman et al., 1996; Keenan & Veldhuis, 1998], and further suggest that E_2 levels fluctuate as a result of testicular steroidogenesis. In particular, E_2 in male *C. kuhlii* appears to be produced in measurable amounts by the testes and/or rapid peripheral conversion of gonadally produced testosterone. Androgens are converted to estrogen by aromatase in the peripheral tissues as well as the central nervous system in mammals [e.g., Truman et al., 1991; Zumpe et al., 1993; Zumpe & Michael, 1994].

Although we did not evaluate hormone levels of castrated male *C. kuhlii*, we observed that urinary levels of T and E_2, but not CORT, were significantly lower among castrated than intact male common marmosets (*C. jacchus*), further suggesting that gonadal activity influences E_2 levels in male marmosets. The data on castrate vs. intact male marmosets do suggest that hormone sources other than the gonad may contribute to E_2 production and secretion, since levels of E_2 were reduced approximately 50% after castration, while levels of T were dramatically reduced 10-fold by castration. Nonetheless, the testis appears to be a source of significant amounts of E_2.

Levels of circulating CORT often increase in mammals after exposure to stress [e.g., Sapolsky, 1986, 1991; O'Byrne et al., 1988, 1989; Sapolsky et al., 2000]. However, we failed to see such increases in plasma CORT levels among male *C. kuhlii* in response to the stressful components of our study, possibly because blood samples were not collected until males had already been exposed to the stressful conditions of the experiment. Alternatively, increases in plasma CORT secretion arising from stress may have been masked among males in our study. Rhythmic circadian declines in CORT levels are common in primates during the middle of the day [Lacerda et al., 1973; Martensz et al., 1987; Smith & Norman, 1987; Bercovitch & Clarke, 1995; Coe & Levine, 1995], and stress-related increases in plasma CORT may have counterbalanced these circadian declines, resulting in no net change in CORT levels among males in our study.

In contrast to plasma CORT, urinary concentrations of CORT increased among male *C. kuhlii* after treatment with GnRH or saline, and returned to near pretreatment baseline levels on the day after the experiment (Table III), supporting the notion that stressful elements or our procedures activated the HPA axis in males. Smith and French [1997] similarly observed stress-induced increases in urinary concentrations of CORT in *C. kuhlii*. Post-treatment increases in CORT were associated with decreases in T and E_2 among saline-treated males in our study. Activity along the HPA axis often suppresses activity along the HPG axis [Sapolsky et al., 2000]. Thus, it remains possible that the increases in CORT we observed in males may have contributed to the post-treatment declines in T and E_2 that occurred in saline-injected subjects. According to this scenario, T and E_2 levels of male *C. kuhlii* are importantly influenced by both HPA and HPG activity.

Our data demonstrate distinct differences in hormonal change between GnRH-treated and control male *C. kuhlii*; however, we observed no correlations between plasma and urinary concentrations of any of the hormones we evaluated in the study. Thus, our data evaluating plasma and urinary hormones are not directly comparable. Plasma was collected from males at two specific time points and represents concentrations of circulating hormones at those particular times. By contrast, post-treatment urine samples were collected over a period of several

hours on the day of the experiment and represent mean hormone levels over a broad time span. Thus, pre- to post-treatment changes in plasma hormone concentrations are not directly equivalent to changes in urinary hormone levels. Our data do not allow us to draw conclusions about the relationship between the secretion and excretion of hormones in *C. kuhlii*. This relationship can be elucidated by future studies which, for example, will examine renal clearance of radioactively labeled hormones injected into subjects. Despite their lack of direct congruity, results of our analyses of both plasma and urinary hormones are consistent with the notion that HPG activity influences E_2 levels in male *C. kuhlii*.

Although we have established a preliminary association between HPG activity and E_2 levels in male *C. kuhlii*, further studies evaluating the interaction between the various elements of the HPG axis, and in particular how E_2 levels vary with levels of LH, FSH, and inhibin, will be needed to more thoroughly illuminate the exact relationship between HPG function and E_2 production and release. Moreover, the possible links between E_2 and reproductive behavior suggested by prior studies of *C. kuhlii* [Nunes et al., 2000, 2001] remain to be elucidated. However, such links are supported by various lines of evidence. The reproductive system of male marmosets is clearly designed to utilize circulating E_2, since the immunoexpression of estrogen receptor-α is found in high concentrations in the testis and excurrent ducts of the male reproductive tract [Fisher et al., 1997]. Further, administration of exogenous E_2 affects the HPG axis in male marmosets in a fashion similar to female-typical patterns (i.e., exogenous E_2 can produce positive-feedback LH responses in intact males [Hodges & Hearn, 1978].

In mammals, E_2 produced locally in the central nervous system appears to have some functions different from those of E_2 reaching the central nervous system via circulation. For example, E_2 produced locally by aromatization, but not E_2 from systemic circulation, is important in mediating masculine sexual behavior in rodents and primates [Bonsall et al., 1992; Zumpe et al., 1993; Zumpe & Michael, 1994; Clancy et al., 1995]. Moreover, E_2 produced by aromatization of T provides negative feedback on gonadotropin secretion in primates, whereas exogenous E_2 may generate positive feedback responses [Ellinwood et al., 1984; Hodges & Hearn, 1978]. Two distinct types of neurons appear to occur in different regions of male rodent and primate brains: those with receptors that bind primarily to E_2 from circulation, and those with receptors that bind to E_2 from circulation as well as E_2 produced locally by the aromatization of T [Michael et al., 1987, 1989; Clancy & Michael, 1994]. Thus, the possibility exists in male marmosets that circulating E_2 produced by the testes has its own distinct functions, and does not merely facilitate the same neuroendocrine effects of T converted locally to E_2 by aromatase.

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