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Suppression of Cortisol Levels in Subordinate Female Marmosets: Reproductive and Social Contributions

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Socially subordinate female common marmosets (*Callithrix jacchus*) have markedly lower plasma cortisol levels than dominant females. Subordinate females also undergo hypoestrogenemic anovulation, and estrogen can elevate glucocorticoid levels. Therefore, we previously hypothesized that this cortisol difference is mediated by rank-related differences in reproductive hormones, probably estradiol. To test this possibility, we characterized the effects of the ovarian cycle and ovariectomy on plasma cortisol concentrations. Beginning in the early follicular phase, basal blood samples were collected from seven cycling female marmosets daily for 16 days and at 2- to 3-day intervals for another 16 days. Samples were collected identically from seven anovulatory subordinate females and seven long-term ovariectomized females. Cortisol levels changed reliably across the ovarian cycle, with levels in the mid- to late follicular, peri-ovulatory, and early luteal phases higher than those in the remainder of the cycle. Cortisol levels of cycling females were significantly higher than those of subordinates at all parts of the cycle, but were significantly higher than those of ovariectomized females only during the midcycle elevation. Unexpectedly, subordinates had significantly lower cortisol levels than ovariectomized females, as well as higher estradiol and estrone levels and lower progesterone and luteinizing hormone (LH) levels. These results confirm that circulating cortisol concentrations are modulated by reproductive function in female marmosets but also indicate that low cortisol levels in subordinate females cannot be attributed simply to hypoestrogenemia. Instead, other factors, such as direct effects of social subordination or suppression of LH levels, contribute to suppression of cortisol in subordinates. **© 1998 Academic Press**

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Measures of hypothalamo–pituitary–adrenal (HPA) activity have been found to correlate with social status in numerous species, with subordinate animals frequently evincing higher adrenal weights, basal glucocorticoid levels, or adrenal responses to stressors than their dominant counterparts (see reviews by Henry, Stephens, and Ely, 1986; Sapolsky, 1995). These findings have led to the widely held view that social subordination is inherently stressful and that the psychosocial stress generated by subordination directly elevates HPA activity. It is becoming clear, however, that this simple socioendocrine model may not adequately explain the diversity of findings on endocrine function and social status. For example, an increasing number of investigations in a growing number of species have failed to demonstrate the expected association of high chronic HPA activity with low social status. Instead, HPA activity may not be systematically correlated with social status (e.g., Mays, Vleck, and Dawson, 1991; Bercovitch and Clarke, 1995; Ziegler, Scheffler, and Snowdon, 1995; Smith and French, 1997) or, in a few cases, may be lower in subordinate animals than in their dominant counterparts (Coe, Mendoza, and Levine, 1979; Schoech, Mumme, and Moore, 1991; Wingfield, Hegner, and Lewis, 1991; Saltzman, Schultz-Darken, Scheffler, Wegner, and Abbott, 1994; Creel, Creel, and Monfort, 1996).

One reason for the absence of a consistent relationship between HPA activity and social status may be that HPA function is modulated by numerous behavioral and physiological variables in addition to psychosocial stress. Moreover, many of these variables may themselves be influenced by social status. For example, physical activity (Coleman, Garland, Marler, Newton, Swallow, and Carter, 1998; Mallick, Stoddart, Jones, and Bradley, 1994), immune function (Hermus and Sweep,

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1990; Gust, Gordon, Wilson, Ahmed-Ansari, Brodie, and McClure, 1991), food intake or nutritional status (Ausman, Gallina, and Hegsted, 1989; Harcourt, 1987), and reproductive function (Kime, Vinson, Major, and Kilpatrick, 1980; Abbott and George, 1991) can all alter HPA activity and may all correlate with an animal's social rank. To understand the relationship between HPA activity and social status in any particular taxon or social group, therefore, it may be necessary to identify the behavioral and physiological variables associated with rank and the ways in which they interact with HPA function.

We have been investigating the relationship between HPA activity and social status in the common marmoset (*Callithrix jacchus*), a small-bodied, cooperatively breeding New World monkey. Social status among females of this species strongly regulates reproduction. Although social groups may contain several adult females, typically only the most dominant female in each group breeds (Epple, 1967; Rothe, 1975; Abbott, 1978; Scanlon, Chalmers, and Monteiro da Cruz, 1988; Stevenson and Rylands, 1988; Saltzman, Schultz-Darken, and Abbott, 1997; but see Digby and Ferrari, 1994). Subordinate females usually undergo suppression of pituitary luteinizing hormone (LH) release and consequent anovulation (Abbott, McNeilly, Lunn, Hulme, and Burden, 1981; Abbott, Hodges, and George, 1988). This socially induced infertility can persist for up to months or years but is rapidly reversed following removal of the subordinate female from her social group (Abbott and George, 1991; Abbott *et al.*, 1988).

We recently demonstrated that subordinate female marmosets also have strikingly lower plasma cortisol concentrations than dominant females (Saltzman *et al.*, 1994; Saltzman, Schultz-Darken, and Abbott, 1996; Abbott, Saltzman, Schultz-Darken, and Smith, 1997; see also Johnson, Kamilaris, Carter, Calogero, Gold, and Chrousos, 1996). However, this difference did not appear to result from social status per se but rather from differential reproductive function (Saltzman *et al.*, 1994). Cortisol concentrations were significantly higher in females undergoing ovulatory cycles than in anovulatory females even when the animals were housed in male–female pairs, as well as when they were housed in social groups as dominants and subordinates. Furthermore, when pair-housed females were introduced into larger, mixed-sex groups, basal cortisol levels (1) dropped markedly within 6–7 weeks only in those animals that ceased ovulatory cyclicity, as a result of becoming subordinate, and (2) increased only in those that commenced regular ovarian cyclicity, as a result of becoming dominant; attainment of dominant or subordinate status without a corresponding change in ovarian function did not systematically alter basal plasma cortisol levels within 6–7 weeks of group formation (Saltzman *et al.*, 1994).

The present study was designed to test our hypothesis that decreased HPA activity in subordinate female marmosets results directly from reproductive suppression rather than from social subordination per se (Saltzman *et al.*, 1994). Specifically, we tested two predictions generated by this hypothesis. First, if plasma cortisol levels are closely regulated by reproductive hormones, then cortisol should change systematically across the ovarian cycle. Second, if the low cortisol levels of subordinate females result from low ovarian steroid levels rather than from a direct effect of social status, then ovariectomized, pair-housed animals should have cortisol levels that are similar to those of subordinate females but significantly lower than those of cycling females. In this experiment, therefore, we characterized plasma cortisol levels across the ovarian cycle in females undergoing normal ovulatory cycles and compared these levels to those of anovulatory subordinates and long-term ovariectomized females.

METHODS

Subjects

Subjects were seven adult female common marmosets (*C. jacchus jacchus*) undergoing ovulatory cycles, seven anovulatory subordinate females, and seven long-term ovariectomized females. Each animal was used in only a single condition except for one animal that was used first while she was undergoing ovulatory cycles and again 16 months later, following ovariectomy; thus, a total of 20 marmosets was used. All subjects were captive-born and were 23–65 months old at the beginning of the study. Cycling, subordinate, and ovariectomized females did not differ significantly in age (41.5 $\pm$ 3.7 vs 37.9 $\pm$ 4.9 vs 50.8 $\pm$ 3.7 months, respectively; mean $\pm$ SEM) or body mass (394.3 $\pm$ 23.2 vs 429.4 $\pm$ 23.6 vs 383.9 $\pm$ 16.9 g, respectively) at the time of data collection. To prevent pregnancies, cycling females were pair-housed with a vasectomized male or, in one case, with a castrated male. Anovulatory subordinate females were housed with a dominant female and one or two gonadally intact males; in four cases, one other subordinate female was also present in the group. Subordinates had not ovulated (see below) for at least 59 days prior to the beginning of data collection and had not had elevated plasma progesterone concentrations ($>$10 ng/ml; see

below), characteristic of the luteal phase of the ovarian cycle, for at least 30 days. Ovariectomized females were pair-housed with an intact male or, in one case, grouped with two intact males and had undergone bilateral removal of the ovaries and Fallopian tubes at least 6 months before the study began (11.1 ± 1.4 months). Ovariectomy was performed by midline incision under Saffan anesthesia (8.1 mg alphaxalone:2.7 mg alphadolone acetate, im; Pitman-Moore, Harefield, Uxbridge, Middlesex, UK). Ovariectomized females had not undergone hormone replacement treatment at any time.

Male–female pairs lived together 5.6 ± 1.5 months before data collection began. We formed larger social groups 7.9 ± 2.5 months prior to the study by releasing three to five unrelated, unfamiliar females and two to four males simultaneously into a large observation room (363 × 212 × 218 cm) containing nestboxes and tree branches. Females' relative social status was assessed on the basis of submissive behaviors observed during the 3 days following group formation: subordinate females performed submissive behaviors to the dominant female cagemate but did not receive submission from her (Saltzman *et al.*, 1994, 1996). Behavioral assessments of dominance status were confirmed by the occurrence of ovulatory cycles in dominant females and anovulation in subordinate females, based on plasma progesterone concentrations in blood samples collected twice weekly (see below). Animals of either sex were permanently removed from their groups at any time if they were involved in persistent aggressive interactions with same-sex cagemates. Groups remained in the observation room for at least 2 weeks following group formation before being moved to smaller home cages. Differences among cycling, subordinate, and ovariectomized females in the total number of cagemates or the number of males present are unlikely to have contributed to differences in cortisol levels, as basal cortisol concentrations do not differ between cycling females that are paired with a male and those that are dominant in larger social groups (Saltzman *et al.*, 1994; Abbott *et al.*, 1997).

During the study, all animals were housed in aluminum and wire mesh cages measuring 61 × 91 × 183 cm, 122 × 61 × 183 cm, or 61 × 61 × 183 cm. Cages were equipped with stainless steel nestboxes, wooden dowels, stainless steel perches, hanging ropes, and rope ladders and permitted animals to see, hear, and smell conspecifics in other cages. Cages were located indoors, with lights on from 0600 to 1800 h, ambient temperature at approximately 27°C, and humidity at approximately 50%. Marmosets were fed Zu/Preem Marmoset Diet (Hill's Pet Products, Topeka, KS) with Provim and Nutra-Plus vitamin/mineral/protein supplements (Nutra-Vet Research Corp., Poughkeepsie, NY), plain yogurt, fruit, vegetables, and miniature marshmallows. Animals were fed once daily at 1300–1500 h, and water was available *ad lib.* Animals were maintained in accordance with the recommendations of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals.*

Blood Sampling and Synchronization of Cycles

To synchronize ovarian cycles, we gave each cycling animal an intramuscular injection of 1.0 μg cloprostenol sodium (Estrumate, Mobay Corp., Shawnee, KS), a prostaglandin F2α analog, on day 0 of data collection, which was timed to occur 13–39 days following the previous ovulation. This treatment causes luteolysis and termination of the luteal phase or early pregnancy (Summers, Wennink, and Hodges, 1985). We also injected six ovariectomized females and two anovulatory subordinate females with the same dose of cloprostenol on day 0, to determine whether cloprostenol directly altered plasma cortisol concentrations.

Blood samples were collected from cycling females daily from day 0 until at least 5 days following ovulation (usually through day 16), and at 2- to 3-day intervals for an additional 16 days. This schedule of blood sampling was used in order to allow accurate assessment of endocrine changes occurring during the follicular and peri-ovulatory periods. To ensure that all groups of animals underwent comparable schedules of blood sampling, blood samples were collected from subordinate and ovariectomized females daily from day 0 through day 16 and at 2- to 3-day intervals from day 18 through day 32.

For blood sample collection, monkeys were rapidly captured from their home cages and briefly restrained in a marmoset restraint tube (Hearn, 1977). Blood (0.2 ml) was collected by femoral puncture into a heparinized syringe and placed on ice. Samples were centrifuged at 2000 rpm for 10 min, and the plasma fraction was extracted and stored at −20°C until assayed. All blood samples were collected between 0830 and 0930 h, several hours after the diurnal cortisol peak (L. M. George and D. H. Abbott, unpublished data). Latency from initial entry into the cage to sample collection was less than 3 min for 98% of samples and averaged 105 ± 1 s. We found no indication that plasma cortisol was elevated in the remaining nine samples, which were collected within 3–6 min from cage entry.

Hormone Assays

Plasma cortisol concentrations for each blood sample were determined in duplicate aliquots by radioimmunoassay (RIA) using an antibody-coated-tube kit (GammaCoat, Incstar Corp., Stillwater, MN; Saltzman *et al.*, 1994). Assay sensitivity at 90% binding was 0.1 ng/tube (1.0 μg/dl) and intra- and inter-assay coefficients of variation (CVs) of a plasma pool assayed in duplicate in each assay (40% binding) were 2.63 and 4.24%, respectively ($n = 5$ assays).

Plasma progesterone concentrations were measured in duplicate aliquots, using a heterologous enzyme immunoassay (Saltzman *et al.*, 1994). Assay sensitivity at 90% binding was 3.6 pg/tube (2.7 ng/ml), and intra- and inter-assay CVs of a marmoset plasma pool (38% binding) assayed in duplicate on each plate were 3.59 and 11.28%, respectively ($n = 13$ assays). Progesterone concentrations were determined in all blood samples collected from each ovary-intact (cycling or subordinate) female and in two samples per week from each ovariectomized female.

Plasma concentrations of immunoreactive LH/chorionic gonadotropin (CG) were measured in all blood samples by a double-antibody RIA using the monoclonal antiserum 518B7 raised in mouse against bovine LH (generously provided by J. Roser, University of California, Davis). The standard reference preparation used was hCG CR-127 (National Hormone and Pituitary Program, Baltimore, MD) with a range of 0.1–5.0 ng/tube (95–18% bound). Potency of CR-127 as determined by radioreceptor and rat uterotropic assays is 14,900 IU/mg. Trace consisted of I^{125}-hCG CR-127 and was iodinated in-house with IODO-GEN reagent (Pierce Chemical Co., Rockford, IL).

For the LH/CG assay, marmoset plasma (25–50 μl for cycling and ovariectomized females, 50 μl for subordinate females) was added to 250 μl assay buffer [phosphate-buffered saline (PBS) with 0.1% gelatin; pH 7.4] and 100 μl antiserum [pipetting concentration: 1:1,000,000 diluted in 5% normal mouse serum (NMS) · assay buffer] and allowed to incubate overnight at room temperature. Each standard curve tube received 50 μl plasma from marmosets pretreated with Antide ([*N*-Ac-d-Nal(2)[1], d-pCl-Phe[2], d-Pal(3)[3], Lys(Nic)[5], d-Lys(Nic)[6], Lys(iPr)[8], d-Ala[10]]-GnRH; 2.5 mg, sc, 2 days prior to blood sample collection) to control for nonspecific plasma interference. Antide is a gonadotropin-releasing hormone receptor antagonist which permits collection of LH-free plasma (Leal, Williams, Danforth, Gordon, and Hodgen, 1988; Lunn, Recio, Morris, and Fraser, 1994); it was synthesized at the Salk Institute (under Contract NO1-HD-0-2906 with NIH) and made available by the Contraceptive Development Branch, Center for Population Research, NICHD. Assay buffer was added to unknown samples as necessary to bring each total plasma volume to 50 μl. Concentrated I^{125}-hCG trace was run through a small 1-in. column of dowex anion-exchange resin (200–400 mesh chloride form; Bio-Rad, Richmond, CA) soaked in 0.01 M PBS buffer (pH 7.8). The dowex column was coated with 1 ml NMS and flushed through with PBS buffer. Trace was diluted with assay buffer and added to each assay tube (100 μl, 40,000 cpms). Following overnight incubation at 4°C, 1 ml second-antibody solution (1% goat anti-mouse gamma globulin (P4 titer; Antibodies, Inc., Davis, CA) in 5% polyethylene glycol (Baker, Phillipsburg, NJ) was added to each tube. Tubes were incubated for 1 h at 4°C and centrifuged for 30 min at 7°C and 1967*g*. The liquid fraction was aspirated, and the tubes were counted in a gamma counter for 5 min.

To validate the LH assay, three different marmoset plasma pools—from cycling females, anovulatory subordinate females, and ovariectomized females, respectively—were assayed for parallelism. In the range of 0.1–1.0 ng/tube, each pool gave a displacement curve parallel to the standard curve (Brownlee, 1960). The recovery of unlabeled hCG added to 25 μl of a marmoset plasma pool was 118.79 ± 9.17% ($n = 12$). Assay sensitivity was 0.1 ng/tube (1.88 ng/ml), and intra- and inter-assay CVs of a quality-control pool assayed in quadruplicate in each assay (25% binding) were 5.96 and 6.88%, respectively ($n = 23$ assays). To further validate the LH RIA, we correlated immunoreactive LH and bioactive LH concentrations in 25 plasma samples from adult female marmosets, using a bioassay modified from that described by Ziegler, Bridson, Snowdon, and Eman (1987; intra-assay CV: 10.60%, inter-assay CV: 15.84%; accuracy: 99.45%; parallel to the standard curve at 0.06–0.2 μl plasma). This yielded a Pearson correlation coefficient between immunoreactive and bioactive LH levels of 0.97 ($P < 0.001$).

Plasma estradiol and estrone concentrations were measured by RIA (Bielert, Czaja, Eisele, Scheffler, Robinson, and Goy, 1976; Sholl, Robinson, and Wolf, 1979) following extraction with 5 ml ethyl ether and celite column chromatography. The estrone assay used antibody 61-335-1 from ICN (Costa Mesa, CA), and the estradiol assay used the E-2 antibody from Holly Hill Biologicals, Inc. (Hillsboro, OR). In the range of 3.2–115 pg/tube (92.7–21.5% bound) for estradiol and 12–219 pg/tube (75.1–11.9% bound) for estrone, a marmoset plasma pool gave a displacement curve parallel to the standard curve (Brownlee, 1960). The recovery of unlabeled estradiol or estrone added to 200 μl

plasma was 102.76 ± 1.71 and 101.72 ± 1.93, respectively ($n = 6$). Assay sensitivity was 3.0 pg/tube (10.0 pg/ml) for estradiol and 3.9 pg/tube (39.65 pg/ml) for estrone. Intra- and inter-assay CVs of a marmoset plasma pool assayed in quadruplicate in each assay (estradiol: 67% binding; estrone: 45% binding) were 6.2 and 12.1%, respectively, for estradiol and 6.2 and 17.5%, respectively, for estrone.

To obtain sufficient plasma volumes (150 μl) from each subject for estrogen assays, we pooled plasma from multiple blood samples from the same animal. For each subordinate and ovariectomized female, we determined the average plasma estradiol and estrone concentration in one to four plasma pools, depending on the volume of plasma available after all other assays had been performed. For each cycling female, we assayed estradiol and estrone in an ''early follicular'' pool (samples collected 5–7 days before the LH surge), a ''midcycle'' pool (4 days before through 4 days after the LH surge), and a ''mid-/late luteal'' pool (5–22 days after the LH surge). Data from one cycling female were omitted from analyses of estradiol levels across the ovarian cycle, because sufficient plasma was not available from this animal to generate an early follicular pool. Similarly, data from one cycling female were omitted from analyses of estrone levels across the cycle, because sufficient plasma was not available to generate a midcycle pool. Estrone concentrations also could not be determined for five animals in the follicular phase due to insufficient plasma volumes; therefore, this phase was omitted from analyses of estrone levels.

Analysis

For all hormones, values that fell below the sensitivity limit of the assay were replaced by the sensitivity limit for analysis and graphing. An LH surge was considered to have occurred if an animal's LH values on a particular day exceeded the mean + 2 standard deviations of that animal's preceding follicular-phase LH values. Ovulation was considered to have occurred on the day after an LH surge, if the surge was followed in 1–3 days by a sustained (≥2 successive days) elevation of plasma progesterone values above 10 ng/ml (Harlow, Gems, Hodges, and Hearn, 1983). Cycling females were not considered to have terminated the previous early pregnancy or luteal phase and entered the follicular phase of a new cycle until plasma LH/CG values dropped to levels typical of the follicular phase (≤5 ng/ml). This occurred 1–4 days following cloprostenol treatment. For each animal, hormonal data collected prior to this time were omitted from analyses.

For comparisons of cortisol concentrations between marmosets, we grouped each animal's cortisol values into 3-day periods and calculated the mean value for the one to three samples in each period. For cycling females, 3-day periods were normalized to the day of the LH surge, which occurred 9.3 ± 0.6 days after treatment with cloprostenol on day 0. For subordinate and ovariectomized females, 3-day periods were normalized to day 9 of blood sample collection. Except where otherwise specified, the means of these 3-day periods were used for analyses of cortisol levels across days.

Statistical analyses were performed using Systat Version 5 for the Macintosh. For each hormone analyzed, the variance was positively correlated with the mean (Rs = 0.88–0.99); therefore, hormone concentrations were log-transformed prior to analysis to reduce heterogeneity of variance and increase normality of the data (Sokal and Rohlf, 1995). Cortisol levels were then subjected to a split-plot analysis of variance, with time (3-day periods) treated as a within-groups factor and condition (cycling vs subordinate vs ovariectomized) treated as a between-groups factor. Post hoc analyses utilized the Fisher least significance difference test for between-groups comparisons and post hoc univariate F tests for within-groups comparisons. Group summary data are presented as back-transformed means ± 95% confidence limits. For all analyses, significance was assessed at the 0.05 level.

RESULTS

Reproductive Hormones

Plasma concentrations of LH, progesterone, estradiol, and estrone from cycling, subordinate, and ovariectomized females are summarized in Table 1. Each cycling female exhibited an LH surge 8–12 days after cloprostenol treatment, followed by a sustained elevation of progesterone above 10 ng/ml beginning 1–3 days later (Fig. 1). LH concentrations in these nonconceptive cycles were higher on the day of the LH surge than in the follicular phase [by definition; overall ANOVA: $F(2,10) = 73.32$, $P < 0.0005$; post hoc test: $P < 0.0005$] or luteal phase ($P < 0.001$) and were higher during the luteal phase than the follicular phase ($P < 0.0005$). Following the LH surge, plasma progesterone concentrations remained above 10 ng/ml for at least 21 days. Plasma estradiol concentrations were lowest during the early follicular phase (5–7 days before the LH surge), highest during the midcycle period (−4 through +4 days from the LH surge), and intermediate during the mid-/late luteal phase (5–22

TABLE 1

Plasma Concentrations (Anti-logs of Means; Upper and Lower 95% Confidence Limits) of Reproductive Hormones in Cycling, Subordinate, and Ovariectomized Females (n = 7 per Group)

	Cycling females				
Hormone	Follicular	Periovulatory	Luteal	Subordinate females	Ovariectomized females
LH	2.79	26.25[a]	5.59	1.89[b]	3.98
(ng/ml)	(3.86, 2.02)	(43.23, 15.94)	(6.69, 4.66)	(2.02, 1.77)	(5.40, 2.94)
Progesterone	3.26	5.96[c]	60.29	3.06	4.35
(ng/ml)	(3.65, 2.91)	(7.95, 4.46)	(80.66, 45.07)	(3.25, 2.89)	(5.82, 3.26)
Estradiol	68.08[d,e]	184.66[f]	167.03[g]	53.17	13.88
(pg/ml)	(194.86, 23.79)	(258.08, 132.13)	(210.54, 132.51)	(84.50, 33.46)	(18.99, 10.15)
Estrone	64.48[e,h]	211.69[f,i]	1406.70[g]	78.89	43.46
(pg/ml)	(167.20, 24.86)	(386.11, 116.06)	(1752.92, 1128.85)	(105.37, 59.06)	(52.02, 36.31)

[a] Day of LH surge.
[b] Data are shown for only six animals due to unusually high LH levels in the remaining subordinate female (see text).
[c] Days of LH surge and ovulation.
[d] Data are shown for only six animals due to insufficient plasma volume for the remaining cycling female.
[e] Days −7 through −5 from the LH surge.
[f] Days −4 through 4 from the LH surge.
[g] Days 5 through 22 from the LH surge.
[h] Data are shown for only two animals due to insufficient plasma volumes for the remaining five cycling females.
[i] Data are shown for only six animals due to insufficient plasma volumes for the remaining cycling female.

days after the LH surge); however, the change across the cycle was not quite significant [$F(2,10) = 3.69$, $P < 0.07$], possibly because we had to pool blood samples collected over several days for the assay. Estrone levels rose progressively across the cycle and were significantly higher during the mid-/late luteal phase than at midcycle ($T = -6.46$, $df = 5$, $P < 0.005$); the change across the entire cycle could not be tested statistically, because follicular-phase estrone values could be determined for only two animals.

LH concentrations in subordinate females (Fig. 2) consistently fell below the sensitivity limit of the assay (96.5% of samples) except for those from one animal, which averaged 45.14 ng/ml. However, bioassay of this female's samples revealed no detectable bioactive LH (≥98% binding). When this animal's data were excluded, mean immunoreactive LH levels of subordinate females were significantly lower than those of ovariectomized females ($P < 0.005$) and those of cycling females during the LH surge ($P < 0.0001$) or the luteal phase ($P < 0.0001$) but not the follicular phase. LH concentrations of ovariectomized females (Fig. 3) tended to be higher than those of cycling females in the follicular phase ($P < 0.08$) but were significantly lower than those of cycling females on the day of the LH surge ($P < 0.0001$) and in the luteal phase ($P < 0.05$).

Plasma progesterone levels of ovariectomized and subordinate females generally remained below 10 ng/ml and showed no sustained elevations (at least two consecutive samples) above this level. Progesterone concentrations were higher in ovariectomized animals than subordinate females or cycling females in the follicular phase [overall ANOVA: $F(2,18) = 4.05$, $P < 0.05$; Fisher tests: Ps < 0.05]. Estradiol levels of both subordinate and ovariectomized females were significantly lower than mean levels of cycling females [$F(2,18) = 35.28$, $P < 0.0001$, Fisher tests: subordinate vs cycling, $P < 0.005$; ovariectomized vs cycling, $P < 0.0001$]. Similarly, estrone levels of subordinate and ovariectomized females were significantly lower than those of cycling females in either the midcycle [$F(2,17) = 17.00$, $P < 0.0005$; Fisher tests: subordinate vs cycling, $P < 0.005$, ovariectomized vs cycling, $P < 0.0001$] or the mid-/late luteal phase [$F(2,18) = 242.17$, $P < 0.0001$; Fisher tests: Ps < 0.0001]. Estradiol ($P < 0.0005$) and estrone ($P < 0.05$) concentrations of ovariectomized females were significantly lower than those of subordinates and frequently fell below the sensitivity limit of the assay (estradiol: 55.6% of pooled samples; estrone: 83.3% of pooled samples).

Cortisol

The omnibus ANOVA of plasma cortisol concentrations revealed significant main effects of groups [$F(2,18) = 8.75$, $P < 0.005$] and time [$F(9,162) = 9.11$, $P < 0.0001$], and a significant groups × time interaction [$F(18,162) = 2.79$, $P < 0.0005$; Fig. 4]. Separate one-way ANOVAs indicated that the groups differed significantly during each of the 10 3-day periods

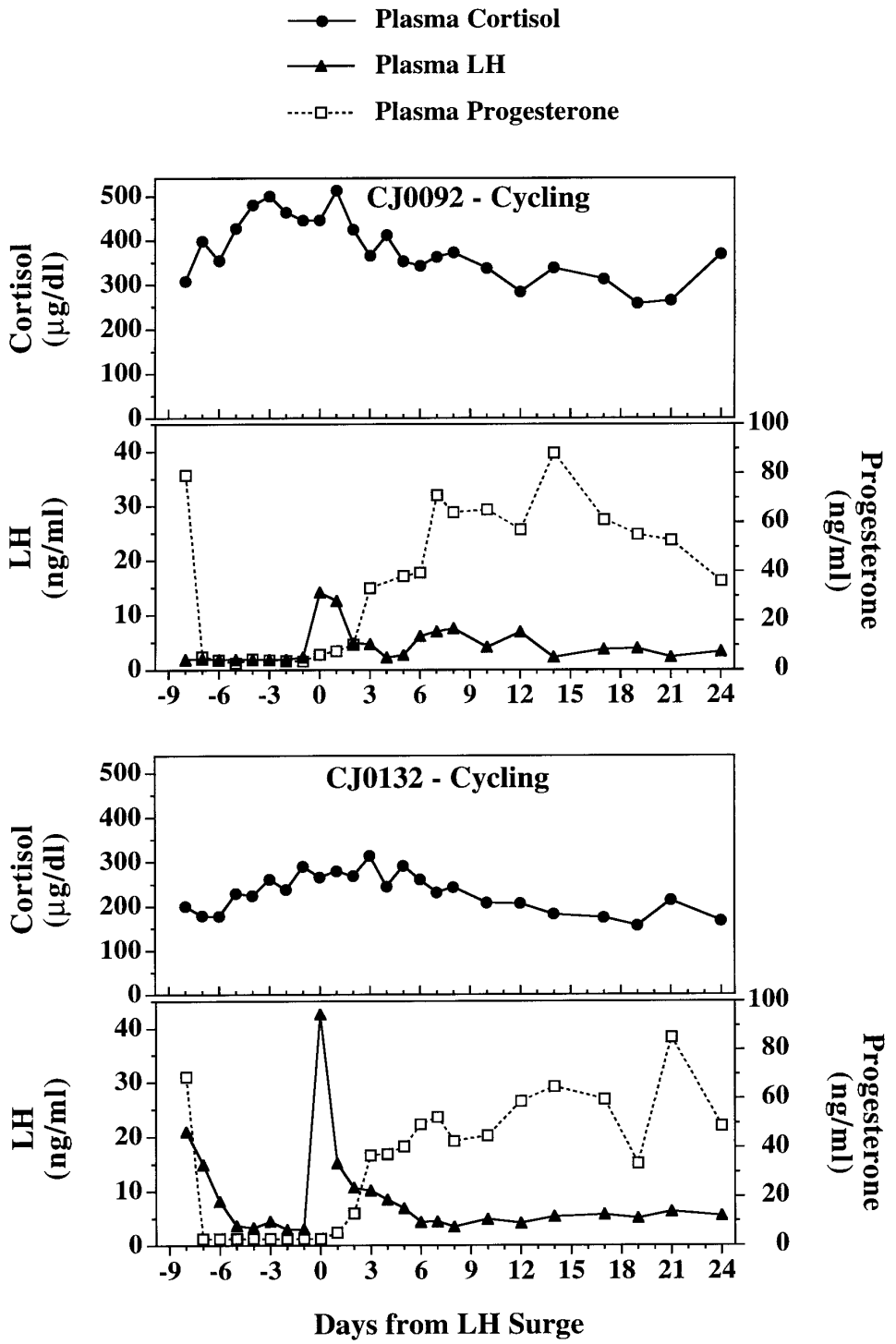

FIG. 1. Plasma cortisol, LH, and progesterone concentrations of two representative female marmosets undergoing ovulatory cycles. Data were normalized to the day of the LH surge.

[$F(2,18) = 5.51–13.04$, Ps ≤ 0.01]. Cortisol levels of cycling females were significantly higher than those of subordinates during each 3-day period (Fisher tests, Ps ≤ 0.005). Cycling females also had higher cortisol levels than ovariectomized animals in each of the 10 3-day periods, and this difference was significant dur-

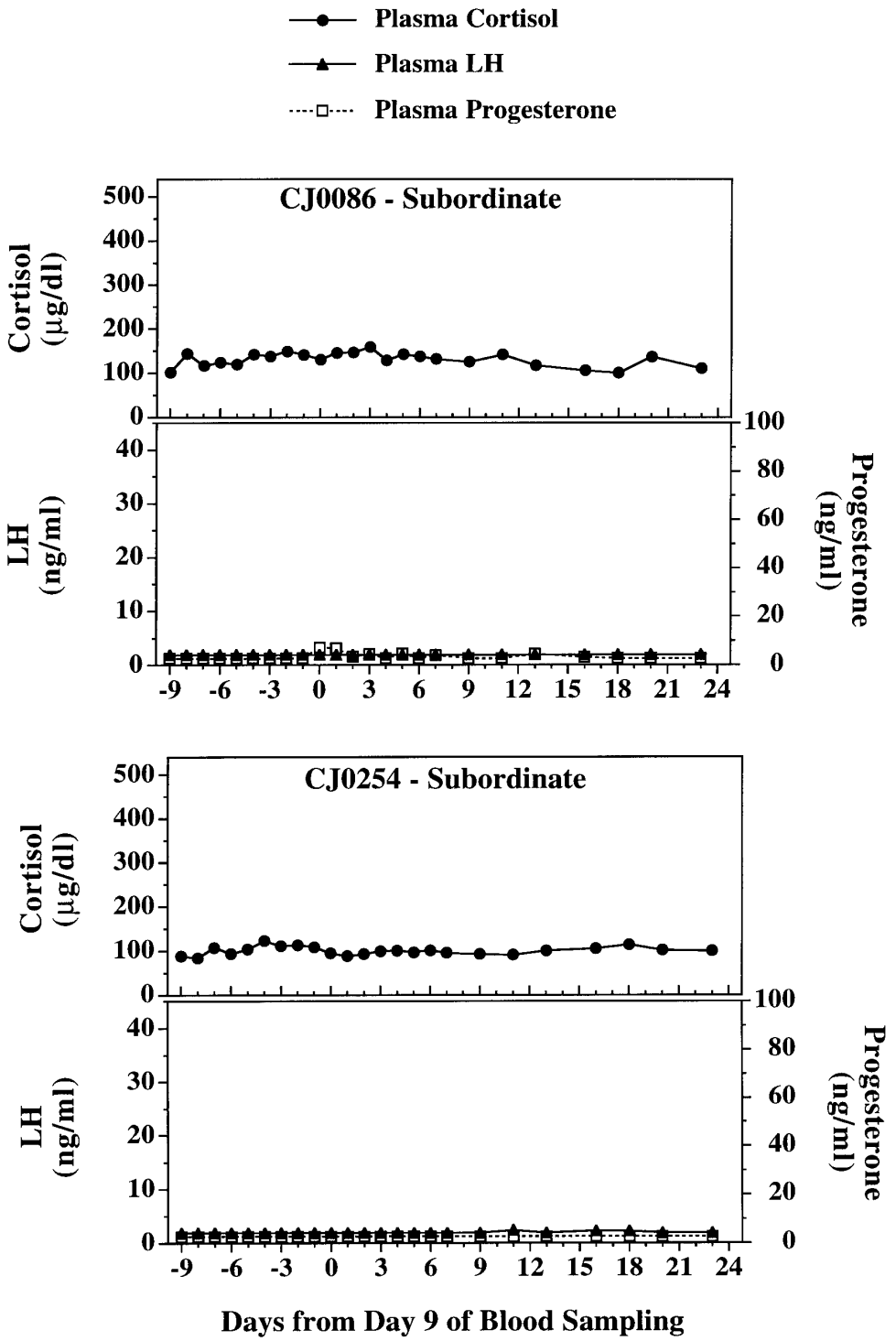

FIG. 2. Plasma cortisol, LH, and progesterone concentrations of two representative anovulatory subordinate female marmosets. Data were normalized to day 9 of blood sampling to facilitate comparison with cycling females.

ing the mid-/late follicular phase (days −4 to −2 from the LH surge, $P = 0.05$), the peri-ovulatory phase (days −1–1, $P < 0.05$) and the early/midluteal phase (days 2–4 and 5–7, $P < 0.05$). Unexpectedly, subordinate females had lower cortisol levels than ovariectomized females in each of the 10 3-day periods, and

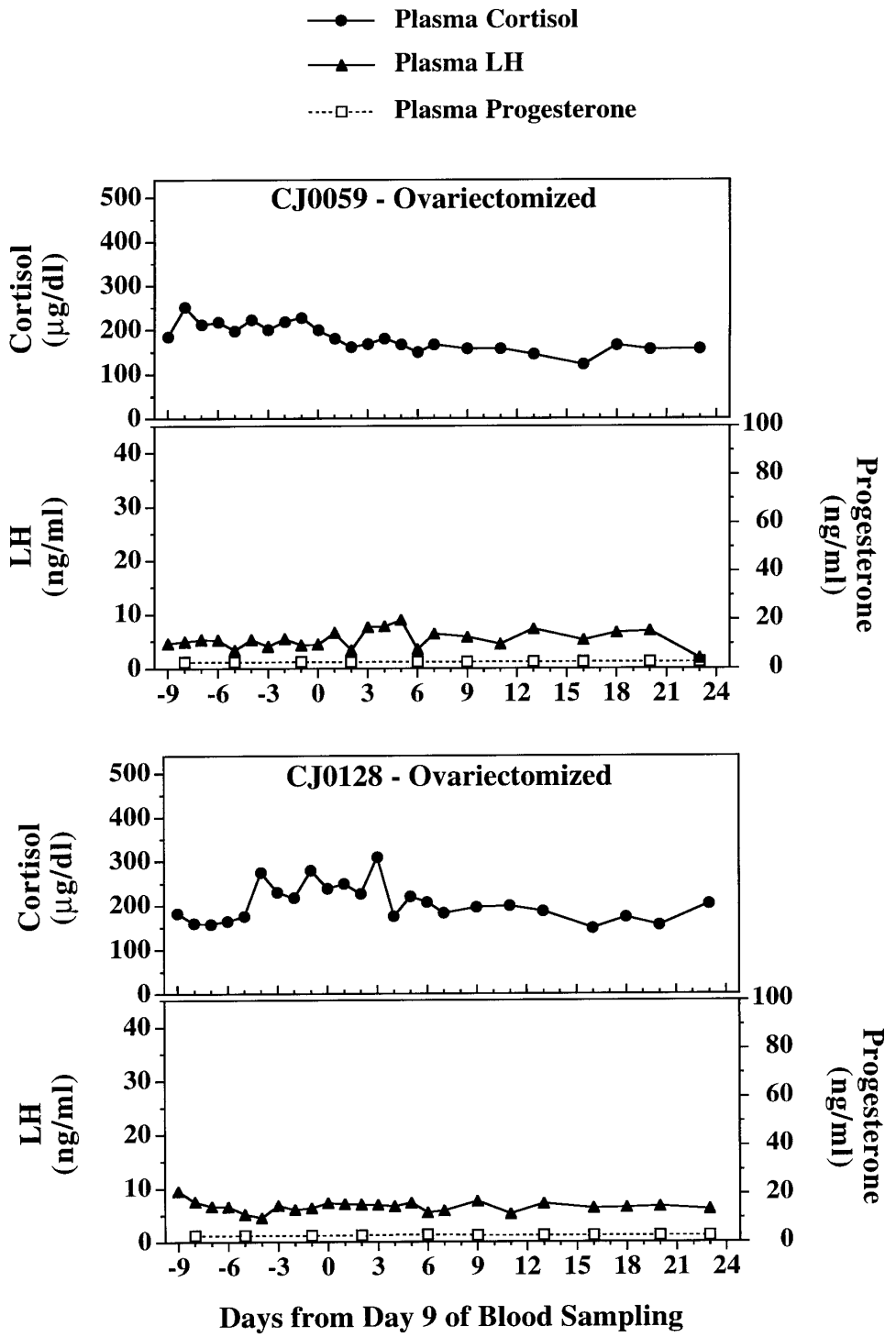

FIG. 3. Plasma cortisol, LH, and progesterone concentrations of two representative ovariectomized female marmosets. Data were normalized to day 9 of blood sampling to facilitate comparison with cycling females.

this difference was significant in seven of the periods (days −1–1, 2–4, 5–7, 8–10, 11–13, 17–19, and 20–22, *P*s ≤ 0.05).

When data from each group of animals were examined separately, plasma cortisol concentrations of cycling females were found to change signifi-

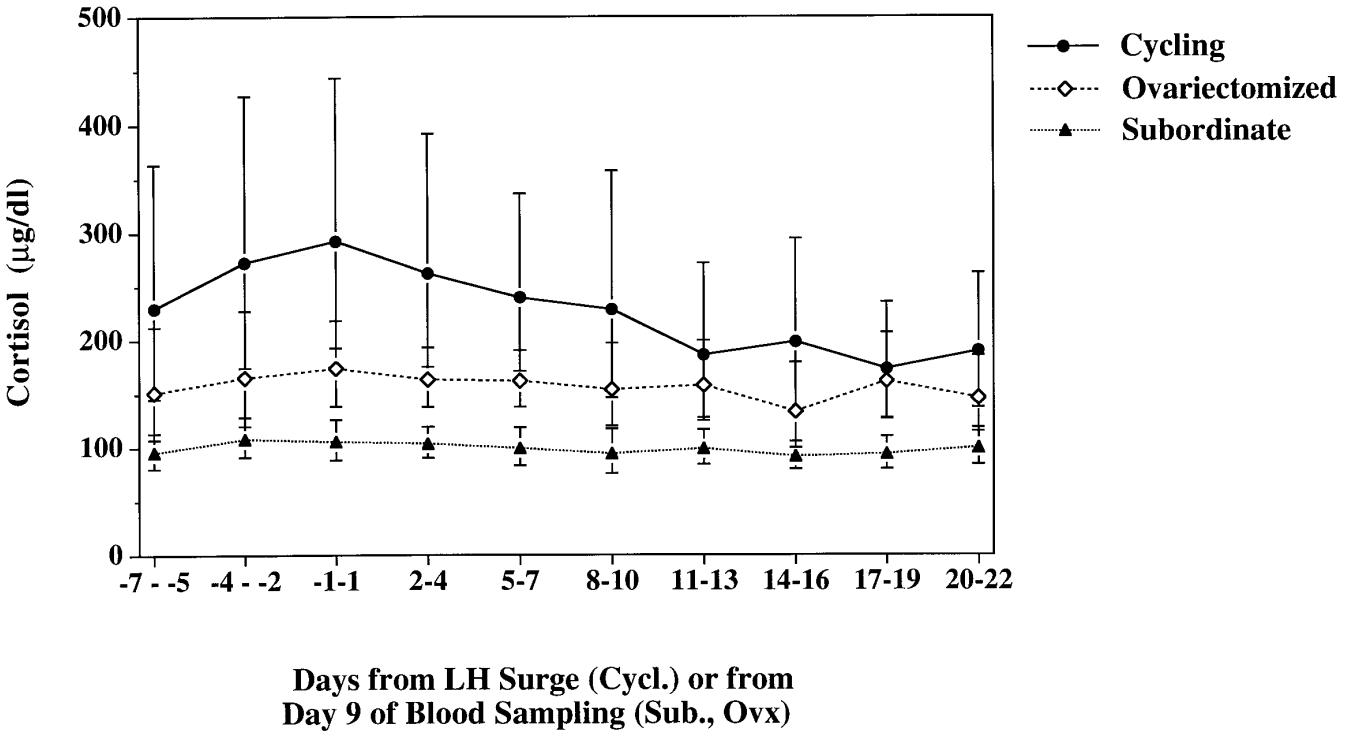

FIG. 4. Plasma cortisol concentrations (anti-logs of means with upper and lower 95% confidence intervals) of cycling, subordinate, and ovariectomized female marmosets ($n = 7$ per group) normalized to the day of the LH surge (cycling females) or day 9 of blood sampling (subordinate and ovariectomized females).

cantly across the ovarian cycle [$F(9,54) = 10.61$, $P < 0.0001$]. Cortisol levels increased from the early to midfollicular phase, peaked during the peri-ovulatory period (days −1–1 from the LH surge), and declined across the luteal phase. Post hoc tests revealed that cortisol values in the mid-late follicular phase (days −4 to −2), the peri-ovulatory period, and the early luteal phase (days 2–4) were significantly higher than those in the mid-/late luteal phase (days 11–13, 14–16, 17–19, and 20–22; Ps < 0.005). Moreover, cortisol concentrations were higher during the mid-/late follicular phase and peri-ovulatory period than during the early follicular phase (days −7 to −5; Ps < 0.05) and were higher during the peri-ovulatory period than during the early/midluteal phase (days 2–4, 5–7, and 8–10; Ps < 0.05). Cortisol levels were therefore significantly higher during the peri-ovulatory period than during all other parts of the cycle except the mid-/late follicular phase. Finally, cortisol levels were lower in the mid-/late luteal phase than in the early/midluteal phase (days 5–7; vs days 11–13: $P < 0.0005$, vs days 17–19: $P < 0.005$, vs days 20–22: $P < 0.05$) and the early follicular phase (days −7 to −5; vs days 14–16 and 17–19: Ps < 0.05).

To determine whether cortisol levels also changed acutely during the midcycle cortisol elevation (late follicular, peri-ovulatory, and early luteal phases), we compared daily cortisol levels from 4 days before through 4 days after the LH surge, the period during which cortisol levels peaked. This analysis confirmed that plasma cortisol levels changed significantly during this time [$F(8,48) = 2.36$, $P < 0.05$]; however, post hoc tests indicated that this effect was due entirely to a decline in cortisol concentrations on day 4 following the LH surge compared to most of the preceding days (vs days −3, 0, 3: Ps < 0.05; vs days −1, 1: Ps < 0.01; vs day 2: $P < 0.005$).

Like cycling females, ovariectomized animals showed a significant change in cortisol concentrations across time [$F(9,54) = 2.15$, $P < 0.05$]. However, pairwise post hoc comparisons did not reveal a clear pattern of differences across time: mean cortisol levels on days 14–16 from the day of normalization (day 9; see Fig. 4) were lower than those on days −4 to −2 ($P < 0.05$), −1–1 ($P < 0.05$), 2–4 ($P = 0.05$), 11–13 ($P < 0.01$), and 17–19 ($P = 0.01$), and mean cortisol levels on days 20–22 were lower than those on days −1–1 and 17–19 (Ps < 0.05). Cortisol levels of subordinate females did not change significantly across the course of the study.

TABLE 2

Pearson Correlation Coefficients for Each Pair of Hormones (Log of Each Animal's Mean Hormone Concentration) in Subordinate ($n = 6$)[a] and Ovariectomized ($n = 7$) Females Analyzed Separately [(S), (O)] or Together [(SO)]

	LH	Progesterone	Estradiol	Estrone	Cortisol
LH	1.0000				
Progesterone	−0.5392 (S)	1.0000			
	−0.5247 (O)				
	0.1692 (SO)				
Estradiol	0.1251 (S)	−0.3565 (S)	1.0000		
	0.1221 (O)	0.4323 (O)			
	−0.6312[b] (SO)	−0.3510 (SO)			
Estrone	0.0284 (S)	−0.5865 (S)	0.4350 (S)	1.0000	
	0.3364 (O)	0.5760 (O)	0.6592 (O)		
	−0.4635 (SO)	−0.2228 (SO)	0.7733[c] (SO)		
Cortisol	−0.5417 (S)	0.4293 (S)	−0.5297 (S)	−0.3493 (S)	1.0000
	0.8870[d] (O)	−0.1966 (O)	0.3917 (O)	0.6562 (O)	
	0.8398[e] (SO)	0.2793 (SO)	−0.5502[f] (SO)	−0.3410 (SO)	

[a] Data were omitted from the one subordinate female with extremely high immunoreactive LH values.
[b] $P < 0.05$.
[c] $P < 0.005$.
[d] $P < 0.01$.
[e] $P < 0.001$.
[f] $P = 0.05$.

To further examine the social and hormonal influences on plasma cortisol levels in noncycling females (subordinate + ovariectomized), we performed a forward multiple regression (P_{in}: 0.05; P_{out}: 0.10) of cortisol on LH, progesterone, estradiol, estrone, and a dummy variable coding for groups (subordinate vs ovariectomized). For each hormone, we used the log of each animal's mean concentration across the entire sampling period; data from the one subordinate female with very high immunoreactive (but not bioactive) LH levels were excluded. Results indicated that LH concentration was the only significant predictor of cortisol concentration (all other P values to enter >0.42; see Table 2 and Fig. 5). The same result was obtained when we excluded the one (ovariectomized) female with the highest mean cortisol concentration. In both cases, the effect was attributable to a strong positive relationship between LH and cortisol in ovariectomized females; subordinate females showed virtually no variation in mean LH levels, possibly because most of their LH values fell below the sensitivity limit of the assay.

Finally, to determine whether cortisol levels were directly altered by cloprostenol, which was injected on day 0 to cause luteolysis, we performed a two-way split-plot ANOVA to compare daily cortisol levels from day 0 through day 5 of blood sampling between acyclic (subordinate + ovariectomized) females that did and did not receive the drug treatment. This analysis did not reveal a significant main effect of cloprostenol treatment or a significant treatment × time interaction. Thus, cloprostenol did not directly alter cortisol concentrations.

DISCUSSION

The results of this study support the hypothesis that circulating cortisol levels in female marmosets are influenced by reproductive function. As predicted, cortisol levels changed systematically across the ovarian cycle and were lower in ovariectomized animals than in females undergoing ovulatory cycles. Unexpectedly, however, we also found that cortisol levels were significantly lower in anovulatory subordinate females than in ovariectomized animals. Thus, hypoestrogenemia alone cannot account for the low circulating cortisol levels in subordinate females, as we had previously speculated (Saltzman *et al.*, 1994). Instead, some other factor(s), such as social subordination per se or suppression of circulating LH levels, must also contribute to suppression of cortisol levels in subordinate female marmosets.

Effect of Social Subordination on Cortisol

We and others have previously shown that anovulatory, socially subordinate female marmosets have

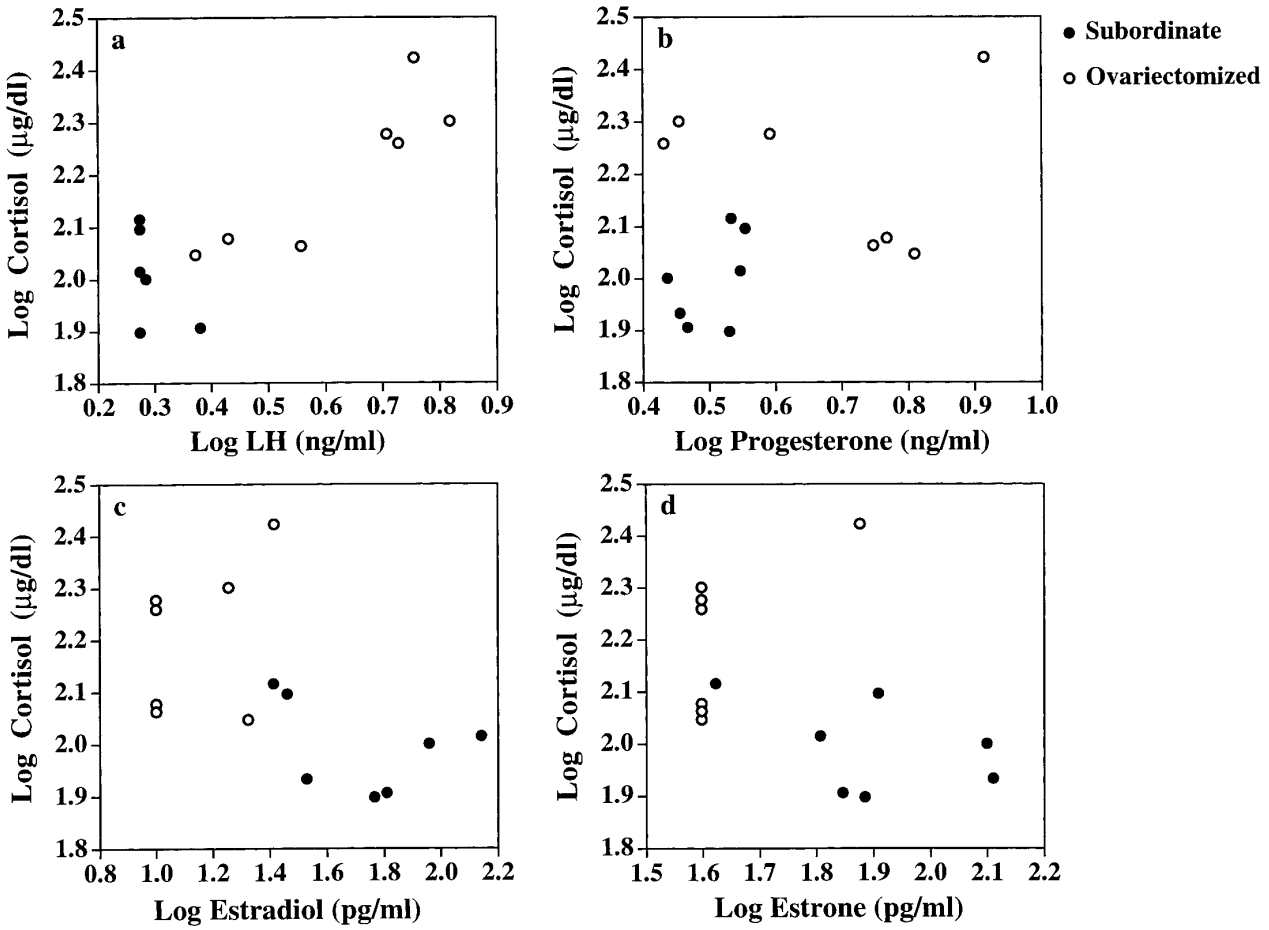

FIG. 5. Relationship of cortisol levels to (a) LH, (b) progesterone, (c) estradiol, and (d) estrone levels in noncycling (subordinate, ovariectomized) female marmosets. Data shown are logs of each animal's mean plasma concentration of each hormone across the entire sampling period. In a multiple regression using data from both subordinate and ovariectomized females, LH levels were the only significant predictor of cortisol levels.

low basal plasma cortisol levels, compared to dominant females undergoing ovulatory cycles, and that this difference is caused by a decline in cortisol levels which occurs within 6–7 weeks after the attainment of subordinate status (Saltzman *et al.*, 1994; Johnson *et al.*, 1996; Abbott *et al.*, 1997). Because estradiol elevates circulating glucocorticoid levels in several primate and nonprimate species (reviewed by Kime *et al.*, 1980; Coe, Murai, Wiener, Levine, and Siiteri, 1986), including the common marmoset (K. M. Kendrick and A. F. Dixson, unpublished data, cited in Kendrick and Dixson, 1985a), we hypothesized that this suppression of cortisol in subordinate females was mediated by suppression of reproductive hormones, particularly estradiol (Saltzman *et al.*, 1994). To test this possibility, in the current study we compared basal plasma cortisol concentrations of anovulatory subordinates with those of females undergoing ovulatory cycles and of long-term ovariectomized animals that were neither dominant nor subordinate to other females. We had predicted that cortisol levels in ovariectomized females would be at least as low as those of subordinates, suggesting that hypoestrogenemia, rather than subordination per se, is the immediate cause of low cortisol levels in anovulatory subordinates.

As expected, the present findings confirmed that anovulatory subordinate females have markedly lower plasma cortisol levels than cycling females. This was a robust effect, with significant differences between groups occurring in all phases of the ovarian cycle and little overlap between groups in cortisol concentrations. However, subordinates also had consistently lower cortisol levels than ovariectomized animals. Because this difference in cortisol was accompanied by an opposite difference in estrogen levels—i.e., subordinates had lower cortisol

but higher estradiol and estrone levels than ovariectomized animals—we conclude that the suppression of cortisol in subordinate females cannot be attributed solely to hypoestrogenemia.

The cause of cortisol suppression in subordinate females below levels seen in ovariectomized animals is not immediately clear. One possibility is that social subordination exerts a direct inhibitory influence on HPA activity independent of its effects on reproductive hormones. Such an effect could involve activation or inhibition of neural circuits which influence release of corticotropin-releasing hormone (CRH) or other adrenocorticotrophic hormone (ACTH) secretagogs, such as vasopressin or oxytocin. We cannot exclude the possibility, however, that social suppression of cortisol levels is mediated by reproductive hormones other than estrogen. Subordinate females had significantly lower plasma levels of both progesterone and LH than did ovariectomized females, and both of these hormones may influence HPA activity (Kime *et al.*, 1980).

Progesterone seems unlikely to account for the observed cortisol difference between subordinate and ovariectomized animals. Although its effects on HPA activity are not well understood, numerous reports suggest that progesterone either suppresses or does not alter circulating glucocorticoid levels (e.g., Ganong, 1963; Rodier and Kitay, 1974; Phillips and Poolsanguan, 1978). Clearly, these effects cannot account for the finding that subordinate females had both lower progesterone levels and lower cortisol levels than ovariectomized (and cycling) females. Moreover, progesterone levels were not highly correlated with cortisol levels in either subordinate or ovariectomized females in the present study.

LH, in contrast to progesterone, seems likely to have contributed to cortisol differences between subordinate and ovariectomized animals. LH effects on HPA activity have not been studied extensively, but *in vivo* experiments have indicated that LH can raise circulating corticosterone levels in rats (Phillips and Poolsanguan, 1978). This effect appears to be mediated at least in part by direct stimulatory effects on the adrenal: LH can stimulate glucocorticoid release from both rat and opossum adrenal tissue *in vitro* (Vinson and Renfree, 1975; Vinson, Bell, and Whitehouse, 1976), and hCG, which is structurally and functionally homologous to LH, increases cortisol release from isolated guinea-pig adrenal cells (O'Connell, McKenna, and Cunningham, 1994). Moreover, LH/CG receptor transcripts and receptor protein have recently been identified in the zona fasciculata of human adrenal glands, the adrenal cortex layer which secretes glucocorticoids (Pabon, Li, Lei, Sanfilippo, Yussman, and Rao, 1996). In contrast to these findings, Kendrick and Dixson (1985b) showed that plasma cortisol levels of ovariectomized, estrogen-primed marmosets were unchanged at 2 and 24 h after an intramuscular injection of gonadotropin-releasing hormone (GnRH), which presumably stimulated LH release. However, the prolonged time course of changes in cortisol following the onset of social subordination in marmosets suggests that LH might alter adrenocortical function over periods of weeks rather than hours (Saltzman *et al.*, 1994, 1996; Johnson *et al.*, 1996; Abbott *et al.*, 1997; D. H. Abbott, W. Saltzman, and N. J. Schultz-Darken, unpublished data). Moreover, because Kendrick and Dixson (1985b) did not measure plasma LH levels, it is unclear over what time course GnRH stimulated LH release in their study. Finally, it is possible that chronic estradiol treatment of ovariectomized animals in Kendrick and Dixson's experiment may have obscured acute adrenocortical responses to GnRH.

In the present study, LH was found to be the best predictor of cortisol levels in subordinate and ovariectomized females together, accounting for over 70% of the variability in cortisol levels. It should be emphasized, however, that this effect was completely attributable to a strong correlation between LH and cortisol levels in ovariectomized females; subordinates showed very little variation in detectable LH concentrations (possibly because most of their LH values were below the assay sensitivity limit) and no correlation between LH and cortisol levels. The relationship between LH and cortisol in subordinate female marmosets therefore remains to be clarified, as do the relative roles of LH suppression and social subordination per se in lowering cortisol in subordinate females. Future studies should attempt to uncouple these two factors by selectively manipulating circulating LH levels in subordinate and ovariectomized animals.

Effect of Ovariectomy on Cortisol

Consistent with the well-known stimulatory effects of estradiol on HPA activity (Kime *et al.*, 1980), ovariectomy causes a reduction in circulating glucocorticoid levels in several species (Kitay, 1963b; Smith and Norman, 1987). In the present study, plasma cortisol levels were lower in ovariectomized females than in intact females in each part of the ovarian cycle, and this difference was statistically significant during the cycling females' midcycle cortisol elevation. These findings are similar to preliminary results of a longitudinal study, which indicated that ovariectomy causes a decline in basal plasma cortisol levels in

female marmosets (Abbott *et al.*, 1997). Compared to the effects of social subordination, however, this decline was modest in magnitude and slow to develop, becoming significant only after 4–6 months from ovariectomy. Moreover, cross-sectional comparisons of basal cortisol levels in long-term ovariectomized females and females undergoing ovulatory cycles failed to reveal a significant difference (Abbott *et al.*, 1997). Together, these findings suggest that pronounced reductions in gonadal hormones, such as those occurring after ovariectomy or social subordination, cause a consistent but subtle dampening of HPA activity in female marmosets.

Effects of the Ovarian Cycle on Cortisol

As predicted, circulating cortisol concentrations in the present study changed significantly across the course of the nonconceptive ovarian cycle. Cortisol levels increased during the follicular phase; peaked during the late follicular, peri-ovulatory, and early luteal phases; and declined in the mid- to late luteal phase. These results are similar to findings from rats (Raps, Barthe, and Desaulles, 1971; Phillips and Poolsanguan, 1978), mice (Nichols and Chevins, 1981), humans (Genazzini, Lemarchand-Béraud, Aubert, and Felber, 1975), and cotton-top tamarins (Ziegler *et al.*, 1995), in which plasma or urinary concentrations of glucocorticoids (and, in some cases, ACTH) increased around the time of ovulation. The significance of these midcycle glucocorticoid elevations is unknown; however, it is intriguing to note that adrenal steroid secretion—specifically, adrenal progesterone release, which appears to be regulated by ACTH—enhances LH secretion during the mid- to late follicular phase in the rhesus monkey (Xiao, Xia-Zhang, Shanen, and Ferin, 1997).

The mechanism of the midcycle glucocorticoid elevation, like its significance, is unclear; however, preovulatory elevations in circulating estradiol levels are likely to be involved. In the present study, estradiol concentrations tended to be higher during the midcycle cortisol elevation than during the remainder of the ovarian cycle. This was also true of LH; however, LH was elevated for only 1–2 days in each animal, whereas the midcycle increase in cortisol persisted for approximately 8–9 days. Moreover, estradiol is known to increase circulating glucocorticoid levels in rodents and primates through a variety of actions on the HPA axis, such as enhancing CRH gene transcription in the hypothalamus (Vamvakopoulos and Chrousos, 1993), elevating synthesis and CRH-stimulated release of ACTH by the pituitary (Kitay, 1963b; Coyne and Kitay, 1969), increasing basal and stress-induced glucocorticoid release by the adrenal (Kitay, 1963a; Coe *et al.*, 1986; Burgess and Handa, 1992), raising corticosteroid-binding globulin (CBG) levels in the blood (Sandberg and Slaunwhite, 1959; Coe *et al.*, 1986), and altering hepatic metabolism of glucocorticoids (Kitay, 1963a; Pepe, Johnson, and Albrecht, 1982). Notably, although estrogen-stimulated increases in CBG levels may play an important role in mediating estrogenic effects on HPA activity in other species (e.g., Sandberg and Slaunwhite, 1959; Coe *et al.*, 1986), this mechanism is unlikely to be important in marmosets. Marmosets have extremely low CBG levels, so that virtually all plasma cortisol circulates unbound or loosely bound to albumin (Klosterman, Murai, and Siiteri, 1986; Pugeat, Chrousos, Nisula, Loriaux, Brandon, and Lipsett, 1984; Robinson, Hawkey, and Hammond, 1985).

While estrogen is likely to mediate the midcycle elevation of cortisol levels in marmosets, it cannot account for the entire cortisol pattern that we observed across the ovarian cycle: cortisol concentrations were lower in the mid- to late luteal phase than in the early follicular phase, whereas estradiol and estrone showed the opposite pattern. This suggests that another hormone(s) may have contributed to the luteal-phase decline in cortisol. One possible candidate is progesterone. As described above, some evidence suggests that progesterone can inhibit corticosterone secretion in the rat (e.g., Ganong, 1963; Rodier and Kitay, 1974). However, a strong inhibitory effect of progesterone on cortisol levels in this species would be inconsistent with the finding that ovariectomized females had higher levels of both progesterone and cortisol than subordinate females in this study. Another possibility is that the luteal-phase decline in cortisol is mediated by testosterone. Although we did not measure testosterone in the present study, Kendrick and Dixson (1983) found that circulating testosterone concentrations were significantly higher in the luteal phase than in the follicular phase of the ovarian cycle in common marmosets, and androgens can inhibit HPA activity in some species (Ganong, 1963; Kime *et al.*, 1980).

HPA Activity, Social Status, and Breeding Systems

In conclusion, the present findings, together with results of previous studies (Saltzman *et al.*, 1994, 1996; Johnson *et al.*, 1996; Abbott *et al.*, 1997), establish that social subordination can result in chronic suppression of circulating glucocorticoid levels in female marmosets. This is clearly at odds with the many studies

demonstrating chronically elevated HPA activity in subordinate animals in a variety of species (e.g., olive baboon: Sapolsky, 1982; cynomolgus macaque: Shively, Laber-Laird, and Anton, 1997; tree shrew: von Holst, 1985; sugar glider: Mallick *et al.*, 1994; rat: Barnett, 1955; mouse: Schuhr, 1987; green anole lizard: Greenberg, Chen, and Crews, 1984). Recently, however, an increasing number of studies have found that subordinate animals have circulating or excreted basal glucocorticoid levels that are either similar to (cotton-top tamarin: Ziegler *et al.*, 1995; black tufted-ear marmoset: Smith and French, 1997; Harris' hawk: Mays *et al.*, 1991) or, as in the present study, lower than those of dominant animals (African wild dog and dwarf mongoose: Creel *et al.*, 1996; Florida scrub jay: Schoech *et al.*, 1991; white-browed sparrow weaver: Wingfield *et al.*, 1991). Interestingly, many of the species which do not show elevated HPA activity in subordinates exhibit singular cooperative breeding, in which reproduction is restricted to one dominant pair per social group and subordinates serve as nonreproductive ''helpers'' (Solomon and French, 1997). Although this apparent correspondence between cooperative breeding and glucocorticoid suppression in subordinates remains to be confirmed, it suggests that the reproductive demands and constraints on individuals may have important and unexpected consequences for the relationship between social status and HPA activity.

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