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Social and Reproductive Influences on Plasma Cortisol in Female Marmoset Monkeys

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SALTZMAN, W., N. J. SCHULTZ-DARKEN, G. SCHEFFLER, F. H. WEGNER AND D. H. ABBOTT. *Social and reproductive influences on plasma cortisol in female marmoset monkeys.* PHYSIOL BEHAV **56**(4) 801–810, 1994.—Subordinate female common marmosets (*Callithrix jacchus*) undergo ovulation suppression and exhibit low plasma cortisol levels compared to the dominant, breeding female. To determine whether this cortisol difference is mediated by the differential reproductive consequences of social status, we monitored plasma progesterone and cortisol in 32 adult female marmosets while they were housed in heterosexual pairs, during the first 3 days of heterosexual group formation, and while animals were housed in established social groups. Cortisol levels prior to group formation were significantly higher in females exhibiting cyclic ovulatory activity than in anovulatory females but were not predictive of social status. Subsequently, when animals were housed in established social groups, dominant (cyclic) females had significantly higher cortisol levels than did subordinate (anovulatory) females. Cortisol levels differed between the pre and postgroup formation conditions only in animals that underwent a corresponding onset or termination of ovulatory cyclicity. Cortisol differences between dominant and subordinate female marmosets therefore appear to be associated with differences in reproductive function rather than with social status per se.

Marmoset *Callithrix jacchus* Cortisol Progesterone Ovulation Anovulation Reproductive suppression Reproduction Dominance Group formation Aggression

ACTIVITY of the hypothalamo–pituitary–adrenocortical (HPA) axis is known to be influenced by social status in a broad variety of mammalian species. Circulating glucocorticoid levels in both dominant and subordinate individuals are typically elevated during periods of hierarchy formation or social instability (14,24,25,38–40,54). Moreover, in established social groups, measures of HPA function, such as plasma glucocorticoid concentrations or adrenal weight, are frequently greater in subordinate individuals than in their dominant counterparts [(7,18,53,57), but see (15)]. These findings have generally been interpreted to mean that formation or disruption of dominance relationships is stressful, and that socially subordinate animals are subjected to greater psychosocial stress than are dominant individuals.

Common marmosets (*Callithrix jacchus*) are small, New World monkeys in which social status strongly regulates reproductive function. In both wild and captive groups, subordinate females typically fail to breed and instead help to provide care for the offspring of the dominant female [(1,21,49,56,61), but see (19)]. Studies of captive marmosets have demonstrated that subordinate females are anovulatory as a consequence of inadequate pituitary release of luteinizing hormone (LH) and, presumably, impaired hypothalamic secretion of gonadotropin-releasing hormone (GnRH) (5,6) These endocrine consequences of subordination are both rapid and reversible: when a cycling female is introduced into a social group in which she becomes subordinate, plasma LH drops to very low levels in 1–4 days and ovulatory cycles soon cease. Conversely, upon removal from the social group, anovulatory subordinate females show an elevation of plasma LH levels within a few days and usually ovulate within 2–3 weeks (3,5).

Although the anovulatory condition of subordinate female marmosets has frequently been attributed to psychosocial stress, subordinate females have not been shown to have higher circulating cortisol levels than dominant females (6). In fact, recent studies of captive marmosets in established groups have found that plasma cortisol concentrations are higher in dominant females than in subordinates (23,33). However, because subordinate social status in female marmosets is regularly accompanied by anovulation, and because ovarian hormones alone can alter HPA activity in a number of species (35), it remains unclear whether rank-related differences in plasma cortisol concentrations in female marmosets result from social status per se or from differences in reproductive function between dominant and subordinate females, independent of their social status.

The present study was designed to clarify the effects of social status, reproductive function, and group formation on circulating

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cortisol levels of adult female marmosets. Previous studies have not evaluated reproductive function in relation to HPA activity or have focused exclusively on females in established social groups, in which reproductive function is confounded with social status. In this study, therefore, we evaluated basal plasma cortisol concentrations as well as ovarian activity while animals were housed in heterosexual pairs, during the first 3 days of introduction into a newly formed, mixed-sex social group, and while females were housed in established social groups.

METHOD

Subjects

Subjects were 32 captive-born, adult female common marmosets (*Callithrix jacchus*) between the ages of 16 and 54 months (25 ± 2 months, mean ± SEM). At least 12 days (58 ± 10 days) prior to commencement of the experimental procedures, each subject was pair-housed with an adult male conspecific and was preassigned to one of eight social groups. The four females assigned to the same group were unrelated and had not lived together previously, with the exception of two females that had been housed together in a group of six juveniles for 4 weeks, 8 months prior to the beginning of data collection.

Throughout the study, animals were housed in stainless steel cages containing perches and nestboxes, allowing visual, auditory, and olfactory contact with marmosets in other cages. Male–female pairs occupied cages measuring approximately 89 cm wide × 85 cm deep × 85 cm high or 75 cm wide × 70 cm deep × 69 cm high. Social groups were formed in a complex of three adjoining cages (88 cm wide × 85 cm deep × 86 cm high each) separated by partial partitions, allowing monkeys to temporarily escape from physical and visual contact with one another. Each group either was housed in this complex for the remainder of the experimental procedures or was moved to a new home cage (two adjoining cages, each measuring 89 cm wide × 85 cm deep × 85 cm high, or a single cage measuring 61 cm wide × 91 cm deep × 183 cm high) at least 2 weeks following group formation. All cages were located indoors, with lights on from 0600–1800 h, ambient temperature maintained at 26.7°C, and relative humidity at 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), bread, fruit, and miniature marshmallows. Monkeys were fed once daily at 1300–1500 h, and water was available ad lib.

Design

We collected data from the four subjects assigned to each group during three experimental phases: pregroup formation, group formation, and postgroup formation. During the pregroup formation phase (~2 weeks), each subject was housed with an adult male, and basal blood samples were collected for determination of baseline plasma cortisol concentrations (see below). The group formation phase (3 days) commenced 2–7 days following conclusion of the pregroup formation phase procedures. The four female subjects and four adult males (that were unfamiliar to one another and had not been housed with any of the female subjects for at least 4 months) were released together into a new home cage, and behavior, attainment of social status, and plasma cortisol levels were monitored for 3 days (see below). The postgroup formation phase (~3 weeks) commenced 5 weeks after group formation. Established social groups were observed to confirm the relative social status of each female, and basal blood sampling was then conducted as in the pregroup formation phase. In both the pre- and post-group formation phases, each female subject additionally underwent three stranger-encounter tests, in which she was permitted to interact across a wire mesh for 15 min with an unfamiliar breeding female; methods of these tests are summarized elsewhere (52).

Formation of Social Groups

We formed social groups by releasing the four preassigned female subjects and four adult males simultaneously into a new home cage. Each monkey's ear tufts had been previously colored to facilitate identification of individuals. Groups were formed between 0930–1240 h. For the subsequent 2 h, occurrence of social interactions, including agonistic, affiliative, and sexual behaviors, was recorded on audio tape by three observers. One hour of behavioral data was similarly collected by two observers approximately 24 h (day 2) and 48 h (day 3) following group formation. At approximately 0900 h on days 1, 2, and 3 of group formation, basal blood samples were collected from all individuals in the group for determination of plasma cortisol levels. Following blood sample collection on days 2 and 3, all animals were inspected for wounds, treated as necessary, and returned to the group formation cage. Individuals were permanently removed from the group at any time following group formation if they received persistent aggression from higher-ranking animals of the same sex.

Dominant or subordinate status was assigned to females on the basis of directionality of submissive behaviors exhibited. Submissive behaviors used for these determinations included squeal ["ngä" (22)], facial grimace (2), and ear tufts flatten (60). Aggressive behaviors included vocal threat ["chatter" (22); "erh-erh" (60)], attack (2), cuff (60), snap-bite (60), genital present (2), ear tufts flick ["tufts flick stare" (60)], and frown (60). Male and female marmosets form separate intrasexual hierarchies in mixed-sex groups, and intersexual agonism occurs relatively infrequently (4,21,49); therefore, only intrasexual interactions were analyzed. Because some subordinate females, in two groups, did not interact agonistically with all other subordinate females prior to removal of animals from the groups, each female was assigned dominant or subordinate status, rather than a numerical rank. The dominant female was defined as the female that received submission from each of her three female groupmates, and did not submit to other females, in the last 30-min period in which she was observed to interact agonistically with each other female. These initial status designations were used for analyses of plasma cortisol concentations prior to and during group formation. However, designation of social status for analyses of cortisol levels and behavior of females in established groups was based on behavioral data collected 5 weeks following group formation; differences between the pregroup formation, group formation, and postgroup formation phases in number of dominant and subordinate females are described below.

Blood Sample Collection, Hormone Assays, and Termination of Pregnancies

Basal blood samples were collected from each subject on 2 days during the pregroup formation phase and again on 2 days during the postgroup formation phase. On each of these days, an initial sample was collected at approx. 0900 h, after which the female was returned to her home cage; a second sample was collected at approx. 1145 h. For each animal, at least 6 days elapsed between successive days of basal blood sample collection. In addition to blood samples used for cortisol determination, we collected blood from all subjects twice per week throughout

the study, and for at least 7 weeks prior to group formation, for plasma progesterone determination to assess ovarian activity.

Monkeys were manually captured and briefly restrained in a marmoset restraint tube (29) while 0.1–0.3 ml blood was collected from the femoral vein into a heparinized syringe. During the group formation and postgroup formation phases, all females in the same group were captured and sampled in rapid succession. For samples assayed for cortisol, the latency from initial entry into the cage to blood sample collection averaged 2 min, 2 s, and was less than 5 min for all but nine samples (96.9%); in these nine samples (mean latency = 8 min, 0 s) we found no evidence that cortisol was elevated by the blood sampling procedure. Blood samples were centrifuged at 2000 rpm for 10 min and the plasma extracted and frozen at −20°C until assayed.

To determine whether blood sample collection at 0900 h alters plasma cortisol levels several hours later, we compared plasma cortisol concentrations in blood samples collected from each of 10 female marmosets at 1145 h under two conditions: once when the animal had been captured at 0900 h, blood sampled, and immediately returned to the home cage, as in our routine blood sampling procedure; and once when the animal had been left undisturbed in the home cage. Cortisol concentrations at 1145 h were not significantly altered by collection of a blood sample at 0900 h (with previous sample: 129.12 ± 12.69 μg/dl; without previous sample: 140.37 ± 21.40 μg/dl).

Direct measurements of marmoset plasma progesterone concentrations were made, without extraction, using a heterologous enzyme immunoassay modified from that described by Munro et al. (42). Briefly, microtiter plates (Nunc-Immuno Plate Maxisorb F96 certified, VWR Scientific, Chicago, IL or Linbro™, EIA II Plus, Flow Laboratories, Inc., McLean, VA) were coated with 200 μl progesterone antibody (generously provided by G. Stabenfeldt, University of California, Davis) diluted 1:52,000 with coating buffer (50 m*M* bicarbonate buffer, pH 9.6). The antibody coating of plate wells continued for 6 h at room temperature and for 2 days at 4°C. The excess antibody was decanted off, and 250 μl phosphate-buffered saline (0.1 *M*, pH 7.0) with 0.1% bovine serum albumin (PBS-BSA) was added to each well prior to storage at −15°C for up to 3 months. Before application of the samples, the plates were brought to room temperature in a humidified chamber for a minimum of 2 h. Samples were prepared by adding 5 μl marmoset plasma to 145 μl diluted charcoal-treated marmoset plasma (CTMP):PBS-BSA (1:30), resulting in a 1:15 plasma dilution. CTMP diluted 1:15 with PBS-BSA was then added to the progesterone standard curve (range: 2.5–500 pg, n = 8). Diluted sample and standards (100 μl) were added to 400 μl enzyme label, progesterone-3-CMO-horseradish peroxidase conjugate (provided by G. Stabenfeldt) diluted 1:200,000 with PBS-BSA. The plate was emptied of PBS-BSA, and 200 μl of the sample:conjugate mixture was added to duplicate wells. The plate was then returned to the humidified chamber for 2 h and subsequently washed five times with 0.15 *M* NaCl, containing 0.05% Tween 20, to remove the unbound sample:conjugate using an automated plate washer. Immediately after washing, 200 μl freshly prepared ABTS substrate [250 μl 40 m*M* 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) and 80 μl 0.5 *M* H_2O_2 added to 24.67 ml 0.05 *M* citrate (pH 4.0)] was added to each well. The plate was incubated in the humidified chamber for 1 h before the reaction was stopped with 50 μl/well stopping reagent (25 ml 0.15 *M* hydrofluoric acid containing 6.0 m*M* NaOH and 50 μl 1.0 *M* EDTA). Absorbence was measured at 410 nm on a Dynatech MR5000 microelisa plate reader (Chantilly, VA). Data were analyzed by weighted least-squares regression analysis (48) and reduced by log-logit transformation to yield progesterone concentrations.

The antibody (R4861) was raised in rabbit against progesterone 11α-hemisuccinate:BSA and cross-reacted (at 50% binding) 47.3% with 11α-hydroxyprogesterone, 0.4% with 17α-hydroxyprogesterone, 0.2% with 20α-hydroxyprogesterone, 2.4% with 20β-hydroxyprogesterone, 39.0% with 5α-pregnane-3,20-dione, 17.0% with 5β-pregnane-3,20-dione, 0.003% with 17β-estradiol, 0.004% with androstenedione, 0.8% with testosterone, and 0.002% with cortisol. Serial dilution of a progesterone-spiked female marmoset plasma pool (1.333–0.010 μl; n = 8) with CTMP (1:15) gave a displacement curve parallel to that obtained with progesterone standards (Sigma, St. Louis, MO) diluted with CTMP (1:15) (13). The recovery of progesterone standards added to 5 μl marmoset plasma pool with low progesterone concentration was 95.4 ± 1.2% (n = 8). The sensitivity of the assay at 90% binding was 4.5 pg, and the intra- and interassay coefficients of variation (47) of a marmoset plasma pool (38% binding) assayed in duplicate on each plate were 2.79% and 12.12%, respectively (n = 126 assays).

Marmoset plasma cortisol concentrations (10 μl of a 1:10 dilution) were measured using an antibody-coated tube radioimmunoassay (RIA) kit, GammaCoat™ (Incstar® Corp., Stillwater, MN). To ensure that the incubation reached equilibrium, the protocol was modified to include overnight incubation at room temperature prior to aspiration of the liquid unbound portion. The protocol was further modified by elevating the range of the standard curve to 0.3–18 ng/tube. Data were analyzed by weighted least-squares regression analysis (48) and reduced by log-logit transformation to yield cortisol concentrations. Serial dilutions of a marmoset plasma pool (10–0.625 μl; n = 5) gave a displacement curve parallel to the standard curve (13). The recovery of cortisol standards added to 10 μl of a 1:10 marmost plasma sample was 100.65 ± 2.56%. Assay sensitivity was 1.0 μg/dl, and intra- and interassay coefficients of variation (47) of a plasma pool assayed in duplicate in each assay (40% binding) were 5.35% and 6.66%, respectively (n = 15 assays).

To provide physiological validation of the cortisol assay, six adult female marmosets were injected with dexamethasone sodium phosphate (American Regent Laboratories, Inc., Shirley, NY; 2 mg, IM) at 0900 h, and plasma cortisol concentrations were measured in blood samples collected 0 and 24 h later. As expected, cortisol concentrations declined significantly, from 129.3 ± 14.8 μg/dl before to 13.3 ± 2.1 μg/dl following dexamethasone treatment, $t(5) = 8.78$, $p < 0.001$. An additional six adult female marmosets were injected with dexamethasone (1.75 mg, IM) at 1700 h and received an injection of human adrenocorticotropic hormone (ACTH; Peninsula Laboratories, Inc., Belmont, CA; 3.5 μg, IV) at 0945 h the next day. Measurement of plasma cortisol in blood samples collected before and after ACTH injection confirmed that ACTH produced a significant elevation of plasma cortisol, $F(4, 20) = 100.66$, $p < 0.001$, from 18.1 ± 3.8 μg/dl at the time of ACTH injection to 80.6 ± 4.3 μg/dl at 30 min postinjection, 129.3 ± 6.4 μg/dl at 60 min, 146.8 ± 8.4 μg/dl at 90 min, and 139.4 ± 10.6 μg/dl at 120 min.

Subjects undergoing ovarian cycles were given an IM injection of 0.75 μg cloprostenol sodium, a prostaglandin $F2_\alpha$ analogue (Estrumate, Mobay Corp., Shawnee, KS), 14–30 days after each ovulation, defined as the day preceding a rise in plasma progesterone concentrations above 10 ng/ml (26). This treatment results in luteolysis and the demise of the luteal phase or early pregnancy (62).

Analysis

To evaluate the relationship between ovarian activity and plasma cortisol during the pregroup formation period, we char-

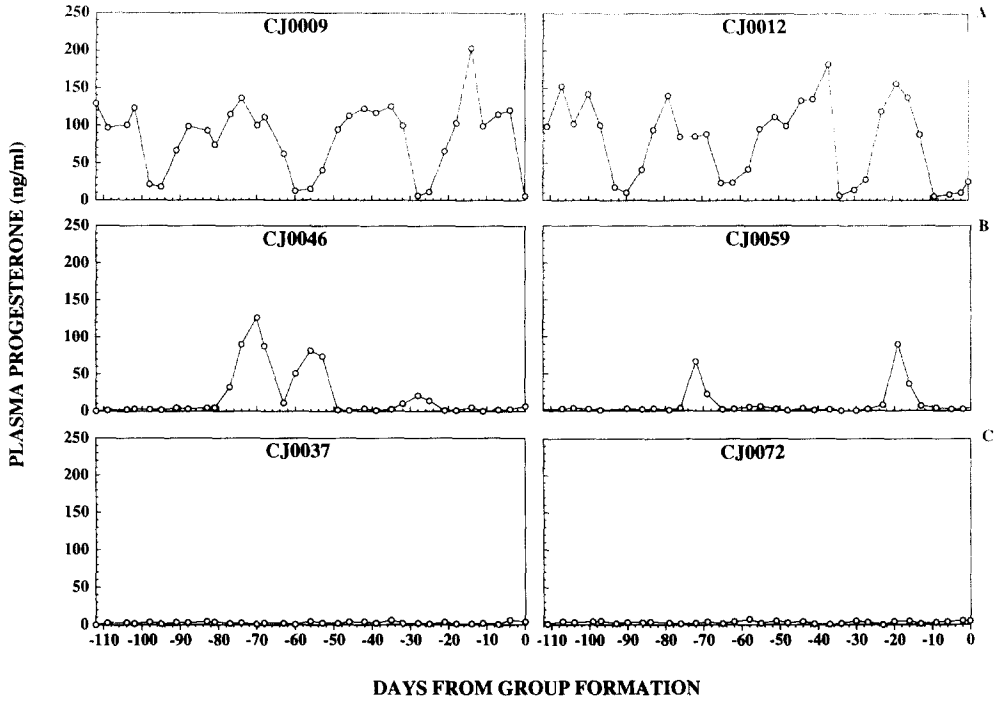

FIG. 1. Representative plasma progesterone concentrations of cyclic (A), oligocyclic (B), and acyclic (C) female marmosets during the 112-day period prior to group formation. Ovarian activity of each animal shown was given the same categorization for the 112-day (three mean cycle lengths + 2 SD) and 37-day (one mean cycle length + 2 SD) periods.

acterized each female as cyclic, acyclic, or oligocyclic on the basis of her plasma progesterone concentrations in semiweekly blood samples, using reproductive endocrine parameters provided by Harlow et al. (26) (follicular phase: 8.25 ± 0.30 days, $n = 56$; luteal phase: 19.22 ± 0.63 days, $n = 48$; complete cycle: 28.63 ± 1.01 days, $n = 19$). Females that showed no sustained (two or more successive samples) elevations of plasma progesterone concentrations above 10 ng/ml were classified as acyclic. Cyclic females exhibited regular, sustained progesterone elevations, with luteal phase (progesterone > 10 ng/ml) durations of at least 11 days (mean − 2 SD) and follicular phase (progesterone ≤ 10 ng/ml) durations of 13 days or less (mean + 2 SD); and oligocyclic females exhibited one or more luteal phase lasting less than 11 days and/or one or more follicular phase lasting more than 13 days. Using these criteria, each female's ovarian activity was characterized for the 37 days (mean cycle length + 2 SD) prior to group formation. For 20 subjects, ovarian activity was also characterized for the 112 days (three mean cycle lengths + 2 SD) prior to group formation; sufficient data were not available for the remaining 12 animals.

Individual animals' cortisol concentrations were averaged for the two basal blood samples collected at each time point (0900 h, 1145 h) within the pre- and postgroup formation phases, respectively. Cortisol data, as well as animals' weights and ages at the time of group formation, were analyzed by analysis of variance (ANOVA) and *t*-tests using the Systat statistical package; post hoc comparisons utilized the Tukey HSD test. Dominance status (dominant or subordinate), ovarian activity (cyclic, oligocyclic, or acyclic), and engagement in wounding aggression (wounded or unwounded) during group formation were treated as between-groups factors; time of basal blood sample, phase of study (pre- or postgroup formation), and day of group formation were treated as within-subjects factors. Significance was assessed at the 0.05 level.

RESULTS

Pregroup Formation

On the basis of plasma progesterone concentrations in semiweekly blood samples, 15 females were categorized as cyclic, eight as oligocyclic, and nine as acyclic during the 37 days (one cycle length + 2 SD) prior to group formation. Of the 20 animals for which progesterone data were available for the 112 days (three cycle lengths + 2 SD) prior to group formation, seven females were classified as cyclic during this period, nine as oligocyclic, and four as acyclic. Oligocyclic females typically exhibited 1–2 brief, low-amplitude progesterone elevations interspersed with prolonged periods (weeks to months) during which progesterone concentrations remained below 10 ng/ml, indicative of anovulation; representative progesterone data for cyclic, oligocyclic, and acyclic females are depicted in Fig. 1. Patterns of ovarian activity for individual animals tended to remain stable over time: plasma progesterone levels of 15 out of 20 females (75%) showed the same pattern in both the 37-day and 112-day periods, whereas two females that were cyclic and three females that were acyclic in the 37 days prior to group formation were classified as oligocyclic over the longer time period. Patterns of ovarian activity in the 37 days prior to group formation were not associated with significant differences in body weight at the time of group formation (cyclic: 358 ± 11 g, oligocyclic: 353 ± 7 g, acyclic: 347 ± 20 g) but did correspond to differences in age, $F(2, 29) = 3.34$, $p = 0.05$: cyclic females (30 ± 3 months, range 19–54 months) were significantly older than acyclic females (19 ± 1 months; range 16–25 months) at group formation ($p < 0.05$,

Tukey), whereas oligocyclic females (25 ± 4 months; range 17–51 months) were intermediate in age and did not differ reliably from the other two groups.

As found in previous studies of marmosets and other New World monkeys (12,17,64), plasma cortisol values were approximately 5–10 times higher than those of Old World primates, including humans. Basal plasma cortisol concentrations of females housed in heterosexual pairs averaged 214.1 ± 21.5 and 171.7 ± 17.0 μg/dl at 0900 and 1145 h, respectively. As anticipated, circulating cortisol levels declined significantly across this period, $F(1, 29) = 17.55$, $p < 0.001$. Basal cortisol levels, as well as the change in cortisol levels across the morning hours, differed significantly between animals in association with ovarian activity during the 37 days prior to group formation [main effect of cyclicity: $F(2, 29) = 7.92$, $p < 0.005$; cyclicity × time interaction: $F(2, 29) = 6.50$, $p < 0.005$] (Fig. 2). Post hoc analyses revealed that cyclic females had significantly higher cortisol levels than acyclic females at both 0900 ($p < 0.001$, Tukey) and 1145 h ($p < 0.01$, Tukey); oligocyclic animals had intermediate cortisol concentrations and did not differ reliably from the other two groups at either time point. Additionally, both cyclic, $t(14) = 4.63$, $p < 0.001$, and acyclic animals, $t(8) = 2.79$, $p < 0.05$, showed significantly lower cortisol concentrations at 1145 h than at 0900 h, whereas oligocyclic females did not. Patterns of ovarian activity in the 112 days prior to group formation were associated with similar differences in plasma cortisol [main effect of cyclicity: $F(2, 17) = 6.13$, $p = 0.01$; cyclicity × time interaction: $F(2, 17) = 6.76$, $p < 0.01$]. Cyclic females had significantly higher cortisol titers than both oligocyclic ($p < 0.05$, Tukey) and acyclic animals ($p < 0.01$, Tukey) at 0900 h and significantly higher levels than acyclic females at 1145 h ($p < 0.05$, Tukey); moreover, all three groups of females showed significant declines in cortisol concentrations across the morning hours [cyclic: $t(6)=4.11$, $p < 0.01$; oligocyclic: $t(8) = 3.43$, $p < 0.01$; acyclic: $t(3) = 7.76$, $p < 0.005$]. Basal cortisol values did not differ reliably between females that later attained the dominant position in their groups (0900 h: 251.5 ± 34.7 μg/dl; 1145 h: 203.0 ± 34.3 μg/dl) and those that attained subordinate status (0900 h: 201.7 ± 26.1 μg/dl; 1145 h: 161.2 ± 19.5 μg/dl).

Group Formation

In all eight groups, a single, dominant female emerged within the 3 days following group formation, as determined by directionality of submission exhibited among females. Overall, dominant females received an average of 19 submissions per hour from each subordinate female cagemate during the first 3 days of group formation (range: 8–51 submissions per hour), whereas they submitted to each subordinate only 0.1 time per hour (range: 0–1 submissions per hour). Conversely, dominant females directed an average of 17 aggressive acts at each subordinate female per hour (range: 2–36 aggressive acts per hour) but were the targets of aggression from each subordinate female only six times per hour (range: 0.3–15 aggressive acts per hour). Dominant females did not differ significantly from subordinates in either age (dominant: 29 ± 5 months, subordinate: 24 ± 2 months) or weight (dominant: 351 ± 11 g, subordinate: 354 ± 10 g) at the time of group formation. Five dominant females had exhibited cyclic ovarian activity in the 37 days prior to group formation, whereas the remaining three were classified as oligocyclic.

In each of three groups, a single subordinate female was removed permanently within the first 24 h due to persistent aggression from the dominant female. Overall, eight subordinates (out of a total of 24 subordinates) from six groups were removed within the first 48 h. Data from these females were omitted from analyses of the adrenocortical response to group formation.

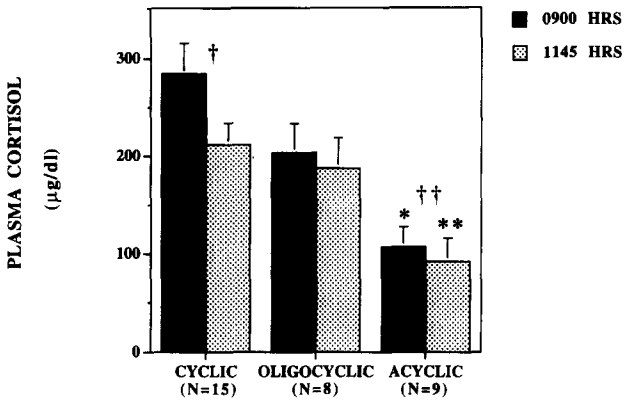

FIG. 2. Basal plasma cortisol concentrations (mean + SEM) of females with cyclic, oligocyclic, and acyclic ovarian activity in the 37 days prior to group formation. *$p < 0.001$ compared to cyclic females, 0900 h; **$p < 0.01$ compared to cyclic females, 1145 h; †$p < 0.001$, 0900 vs. 1145 h, cyclic females; ††$p < 0.05$, 0900 vs. 1145 h, acyclic females.

Dominant and subordinate females showed significantly different changes in plasma cortisol levels across the 3 days of group formation, $F(2, 44) = 6.98$, $p < 0.005$. On day 1, immediately before group formation, cortisol levels did not differ between females that later became dominant and those that became subordinate. On day 2, however, dominant females had significantly higher circulating cortisol concentrations than did subordinates, $t(22) = 3.76$, $p < 0.005$, and this difference persisted on day 3, $t(22) = 4.66$, $p < 0.001$.

These rank-related differences in cortisol appeared to be attributable to engagement in wounding aggression. All eight dominant females, but only six of the 16 subordinates that remained in their social groups, sustained wounds resulting from intrasexual aggression during the first 3 days of group formation, with injuries typically ranging from minor scratches to moderate bite wounds on the face and extremities. Monkeys that were or were not wounded showed different adrenocortical responses to group formation during this time period, $F(2, 44) = 8.46$, $p < 0.001$ (Fig. 3). Females that sustained wounds within the first 48 h following group formation showed a significant elevation of cortisol over basal levels on days 2 and 3 of group formation [$t(13) = 7.42$, $p < 0.001$ and $t(13) = 4.25$, $p < 0.001$, respectively], whereas the 10 subordinate females that were not wounded showed no increase in plasma cortisol levels during the 3 days of group formation. Consequently, although cortisol did not differ reliably among these two groups of animals on day 1, females that engaged in wounding aggression had significantly higher cortisol concentrations than those that did not on day 2, $t(22) = 5.15$, $p < 0.001$, and day 3, $t(22) = 4.76$, $p < 0.001$, of group formation. Among the females that engaged in wounding aggression, cortisol levels of dominant and subordinate animals did not differ significantly. Notably, subordinate females that did not engage in wounding aggression nonetheless engaged in high levels of agonism: they received an average of 11 aggressive acts per hour (range 2–37 per hour) from other females, including seven per hour from the dominant female, and performed an average of 31 submissions per hour (range 11–67 per hour) to other females, including 17 per hour to the dominant female.

As in the pregroup formation condition, plasma cortisol concentrations during group formation differed significantly between females exhibiting cyclic, oligocylic, and acyclic ovarian function in the 37 preceding days, $F(2, 21) = 15.93$, $p < 0.001$: acyclic females had significantly lower cortisol levels (mean ±

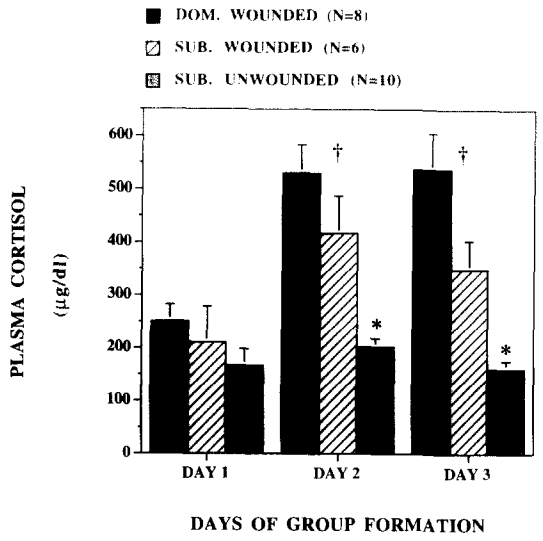

FIG. 3. Plasma cortisol concentrations (mean + SEM) of dominant females (all of which sustained wounds in the first 48 h following group formation) and subordinate females that did and did not sustain wounds, on days 1, 2, and 3 of group formation. *$p < 0.001$ compared to wounded animals; †$p < 0.001$ compared to day 1 (dominant and subordinate wounded, combined).

SEM across 3 days of group formation: 159.1 ± 18.6 μg/dl) than both cyclic (422.7 ± 38.5 μg/dl, $p < 0.001$, Tukey) and oligocyclic females (308.0 ± 40.3 μg/dl; $p < 0.05$, Tukey). However, the change in cortisol concentrations across the 3 days of group formation did not differ significantly as a function of ovarian activity.

Postgroup Formation

In the 5 weeks following group formation, one group was disbanded and six females were removed from their groups due to persistent intrasexual aggression. Postgroup formation data were therefore collected from a total of 17 females (7 dominant and 10 subordinate) in seven established groups, each containing 1–3 males (mean = two males per group).

In six groups, focal-group behavioral data, collected 5 weeks after group formation, confirmed the social status (dominant or subordinate) assigned to females during the initial group formation period. Dominant females in these groups never submitted to other females but received an average of 26 submissions per hour from each subordinate female cagemate (range 4–117 submissions per hour). Frequencies and intensities of aggression were low: dominant females directed an average of one aggressive act per hour to each subordinate female (range 0–3 aggressive acts per hour) and received virtually no aggression in return (mean = 0.1 aggressive acts per hour; range 0–0.3); no fights were observed between females. In each of these six groups, the dominant female exhibited cyclic ovarian activity; subordinate females either remained acyclic or ceased cycling following termination of the ovarian cycle occurring at the time of group formation, no more than 16 days after grouping. In the remaining group, only one instance of submission and one instance of mutual, low-level aggression (ear tufts flick) were scored between the two females during the observations in the fifth week following group formation, and both females ovulated and conceived approximately 14 days after group formation. Because the ranks of these latter two animals could not be confirmed in observations of the established group, their data were omitted from analyses of plasma cortisol concentrations in dominant and subordinate females.

Basal cortisol values of dominant, cycling females in established groups were significantly higher than those of subordinate, acyclic females, $F(1, 13) = 16.30$, $p = 0.001$ (Fig. 4). As in the pregroup formation condition, basal cortisol levels dropped significantly between 0900 and 1145 h, $F(1, 13) = 9.10$, $p < 0.01$; the magnitude of this drop did not differ reliably between dominant and subordinate individuals. A three-way ANOVA (pre- vs. postgroup formation × rank × time) failed to reveal any significant differences between the pre- and postgroup formation conditions, indicating that neither attainment of social status nor membership in a group per se reliably altered cortisol concentrations.

To further clarify the effects of social status and reproductive status on plasma cortisol, we compared pre- and postgroup formation basal cortisol levels for females that had or had not undergone a change in ovarian activity in association with attainment of dominant or subordinate status. Among females that exhibited cyclic ovarian activity in the 37 days prior to group formation and subsequently remained in their social groups as cyclic dominants ($n = 3$), basal cortisol concentrations did not differ reliably between the pre- and postgroup formation conditions (Fig. 5). Similarly, no significant difference was found among females that were initially acyclic and that remained in their groups as acyclic subordinates ($n = 7$). However, among females that were oligocylic prior to group formation and subsequently became cyclic dominants ($n = 3$), cortisol levels were significantly higher following group formation, $F(1, 2) = 91.69$, $p = 0.01$. Only two females that had exhibited evidence of ovarian cyclicity (both oligocyclic) remained in their groups as acyclic subordinates, precluding statistical analysis; nonetheless, their basal cortisol concentrations were strikingly lower in the postgroup formation condition than prior to group formation. Thus, social status of females in established groups was associated with a change in basal plasma cortisol, as compared to the heterosexual-pairs condition, only when attainment of dominant or subordinate status led to a subsequent commencement or termination of ovulatory cyclicity, respectively.

DISCUSSION

Subordinate animals are generally thought to experience pronounced psychosocial stress, resulting from reduced access to

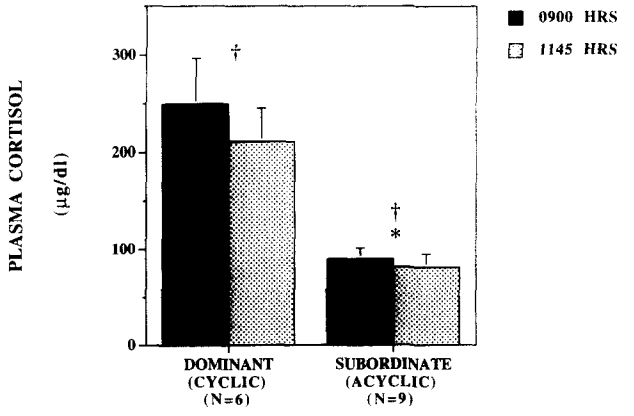

FIG. 4. Basal plasma cortisol concentrations (mean + SEM) of dominant and subordinate females housed in well-established groups. *$p = 0.001$, subordinate compared to dominant (0900 and 1145 h, combined); †$p < 0.01$, 0900 vs. 1145 h (dominant and subordinate, combined).

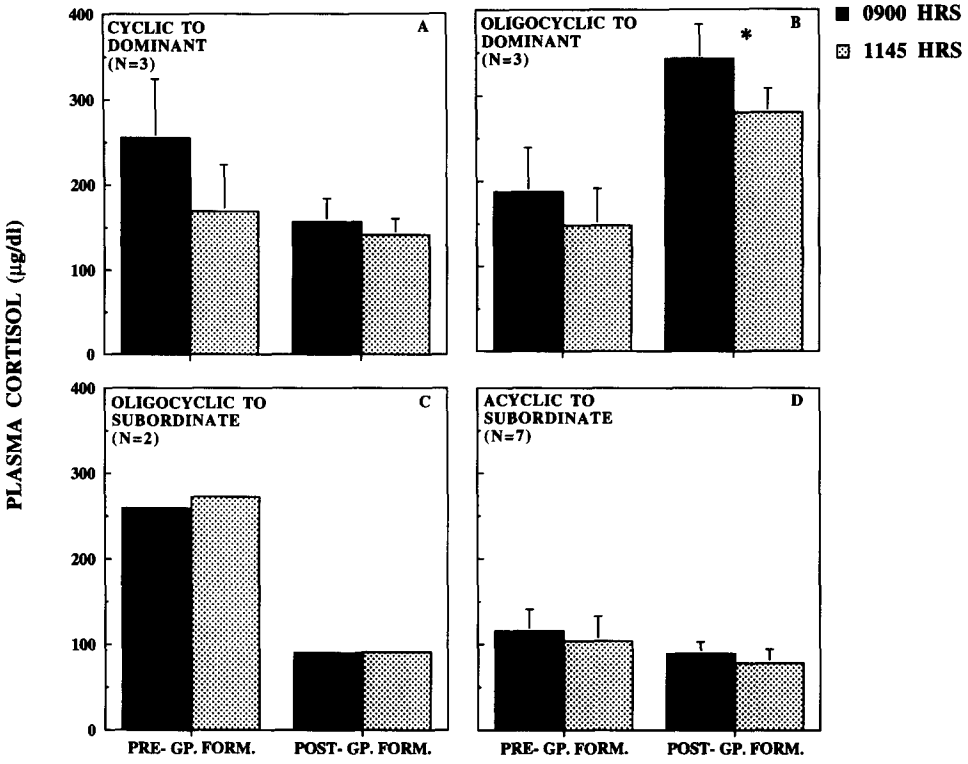

FIG. 5. Basal plasma cortisol concentrations (mean + SEM) of females while housed in heterosexual pairs and in well-established heterosexual groups. (A) Dominant females that exhibited cyclic ovarian activity both before and after group formation; (B) dominant females that exhibited oligocylic ovarian activity before and cyclic ovarian activity after group formation; (C) subordinate females that exhibited oligocyclic ovarian activity before and acyclic ovarian activity after group formation; and (D) subordinate females that exhibited acyclic ovarian activity both before and after group formation. *p =0.01, pre- vs. post-group formation (0900 and 1145 h, combined).

resources as well as from intimidation or harassment by dominant individuals. Consistent with this view, activity of the HPA axis has been found to be elevated in subordinate animals, compared to their dominant counterparts, in many species [reviewed in (31,44,55)]. Among female common marmosets, physiological and behavioral consequences of subordination are particularly distinct. Subordinate females are usually anovulatory, they engage in little or no sexual behavior, they appear to be excluded from heterosexual "pair bonds," and, at least in newly formed groups, they may be subjected to intense aggression from other females, frequently necessitating their removal from the group (2,4,21,49). In fact, in the present study, more than half the subordinate females (14/24, 58%) had to be removed from their groups in the first 5 weeks following group formation as a result of persistent harassment from other females. Nonetheless, the results of this study indicate that attainment of subordinate social status per se does not elevate HPA activity. To the contrary, cortisol levels of subordinate female marmosets that remained in well-established groups were on average 63% lower than those of their dominant counterparts.

The finding of higher basal plasma cortisol concentrations in dominant than subordinate female marmosets is in close agreement with results of two previous studies (23,33). A similar trend was also reported in two earlier articles (2,6) but did not reach statistical significance, possibly due to small sample sizes and greater variability in the times of day at which blood sampling and animal husbandry procedures were performed. However, the present study is the first to demonstrate that this rank-related difference in cortisol can be accounted for by differences in reproductive function between dominant and subordinate females. Cyclic and acyclic females exhibited comparable differences in cortisol both when housed in heterosexual pairs, prior to group formation, and when housed in established heterosexual social groups, in which they had attained dominant (cyclic) or subordinate (acyclic) status. Furthermore, comparisons of hormone levels of individual animals in the heterosexual-pairs and established-groups conditions indicated that cortisol changed reliably only when cyclic ovarian activity commenced, as a result of females becoming dominant, or when ovarian cyclicity ceased, as a result of females becoming subordinate. Attainment of dominant or subordinate status without an accompanying change in ovarian function was not associated with a consistent alteration in basal cortisol levels. Thus, differences in plasma cortisol concentrations of dominant and subordinate female marmosets appear to correspond to rank-related differences in reproductive function rather than to psychosocial correlates of social status, such as stress or arousal.

Not only did social subordination fail to elevate HPA activity among females in established social groups, but initial attainment of subordinate status during the first 3 days of group formation was not closely associated with elevated cortisol at either 24 or 48 h postgroup formation. Instead, only those females that sustained wounds from fighting during this period—including all eight dominant females but only six of 16 subordinate females that remained in their groups through the first 48 h—showed significant cortisol elevations (subordinates that started to receive

persistent wounding aggression were removed from their groups and were not included in analyses of adrenocortical responses to group formation). It is unknown whether these elevations resulted from psychosocial stress associated with fighting and wounding or from other factors such as increased cytokine concentrations or high levels of physical activity, both of which can elevate plasma glucocorticoids in other species (9,32). Furthermore, it is possible that the unwounded animals underwent transient increases in adrenocortical activity or alterations in the diurnal rhythm of cortisol secretion that were not detected due to our blood sampling frequency. Among female squirrel monkeys, for example, plasma cortisol concentrations are significantly elevated 1 h following formation of unisexual dyads, but do not differ from previous baseline values 24 h later and fall to below preceding baseline values after several days (41). More typically, however, social instability, resulting from formation of new social relationships or disruption of existing ones, has been found to stimulate pronounced increases in plasma glucocorticoid concentrations in both dominant and subordinate individuals, and such effects may persist for up to several weeks (14,24,25,38–40). Moreover, in contrast to the present findings, these elevations in circulating glucocorticoids are often independent of engagement in aggression or wounding (11,25,51). Thus, the finding that unwounded subordinate female marmosets showed no significant change in cortisol concentrations following group formation, in spite of their engagement in high levels of agonistic behavior, is particularly surprising and suggests that HPA activity in these animals is not highly sensitive to perturbation by such psychosocial phenomena as formation of new social relationships and attainment of subordinate social status.

In contrast to psychosocial variables, reproductive status was clearly associated with plasma cortisol levels in the present study: cortisol concentrations were dramatically elevated in marmosets undergoing ovulatory cycles compared to anovulatory females, independent of housing conditions and social status. Gonadal steroids have long been known to modulate activity of the HPA axis (36). Estrogen, in particular, acts at multiple sites within the HPA axis to elevate plasma glucocorticoid levels. In rodents, estrogen can alter hypothalamic turnover of corticosteroid-releasing hormone (CRH), stimulate pituitary synthesis of ACTH, increase adrenocortical release of corticosterone, elevate circulating levels of corticosteroid-binding globulin (CBG), and modulate hepatic metabolism of glucocorticoids [reviewed in (35)]. Ovarian steroids similarly elevate HPA activity in primates. In women, plasma cortisol concentrations are elevated by oral contraceptives and by pregnancy (9). Coe et al. (16) demonstrated that estradiol treatment of adult male and female squirrel monkeys stimulated dramatic elevations of plasma cortisol and CBG concentrations within 24 h. Correspondingly, Smith and Norman (58) demonstrated that plasma cortisol levels of ovariectomized rhesus macaques were significantly lower than those of intact females in either the follicular or luteal phase of the ovarian cycle. However, ovariectomized rhesus monkeys exhibited diurnal rhythms in cortisol that were comparable to, although of smaller amplitude than, those in intact, cycling females, similar to our finding that both cyclic and acyclic marmosets showed significant cortisol declines across the morning hours. Thus, ovarian steroids can dramatically elevate basal plasma cortisol concentrations in primates but do not appear to substantially alter diurnal patterning of HPA activity.

Estradiol seems likely to play the principal role in elevating plasma cortisol levels in cycling female marmosets. Exogenous estradiol can significantly elevate plasma cortisol levels in ovariectomized marmosets (34). Moreover, whereas both estrone and progesterone in marmosets are markedly higher during the luteal phase than the follicular phase, estradiol does not vary substantially across the ovarian cycle, with the exception of a brief preovulatory surge [(26,27,30), Scheffler, Schultz-Darken, and Abbott, unpublished data]; correspondingly, a previous study of female marmosets found that plasma cortisol titers do not change significantly across the cycle (23). Plasma cortisol levels also do not differ in women (9) or rhesus macaques (58) in the follicular and luteal phases of the ovarian cycle, during which circulating estradiol levels are comparable but progesterone levels are widely divergent. Finally, estrogen levels of cycling marmosets in both the follicular and luteal phases are higher than those of acyclic, subordinate females (5). It is noteworthy that estrogenic stimulation of CBG is unlikely to significantly alter plasma cortisol levels in marmosets: CBG levels in this species are extremely low, so that virtually all plasma cortisol circulates unbound or loosely bound to albumin (37,45,46).

It is unclear why approximately one-fourth of the marmosets in this study exhibited acyclic ovarian activity while pair-housed with a male, prior to group formation. To some extent, this finding reflects maturational effects, as acyclic females tended to be younger than cyclic animals. However, immaturity cannot entirely account for the high incidence of anovulation. Female marmosets initially show elevations of circulating estradiol into the preovulatory range at approximately 7–10 months of age and undergo their first ovulation at approximately 13–14 months (1). Acyclic animals in the present study were 16–25 months of age at the time of group formation and were therefore within the adult age range. Moreover, several females which were acyclic in the 37 days prior to group formation had ovulated previously and had therefore attained puberty. It is also clear that acyclic females did not simply suffer permanent reproductive dysfunction: eight of the nine females that were anovulatory in the 37 days prior to group formation have subsequently conceived and/or ovulated; the ninth animal has remained as a subordinate in a social group, so that its persistent anovulation is probably attributable to social suppression. Finally, acyclic, singly housed adult females have been described in at least one other colony of captive common marmosets (63), suggesting that transitory periods of anovulation among adult females may represent a relatively common occurrence in this species.

An important goal for future research will be to determine the functional significance of elevated HPA activity in cycling female marmosets. Chronic hypercortisolemia is typically associated with a number of pathophysiological effects, including infertility. In rhesus monkeys and women, for example, Cushing's disease or administration of exogenous glucocorticoids can inhibit hypothalamic release of GnRH, gonadotropin secretion, ovarian steroid secretion, and ovulation (8,20,28,50,59). In contrast to these pharmacological and pathologic elevations of glucocorticoids, however, increased plasma cortisol levels in female marmosets accompany, rather than suppress, reproductive activity, suggesting that these cortisol levels do not represent functional hypercortisolemia but might instead be physiologically appropriate for reproductively active marmosets. Furthermore, the glucocorticoid receptor of Callitrichid monkeys (marmosets and tamarins) has low affinity for cortisol (10), suggesting that females undergoing ovarian cyclicity may be buffered, to a large extent, from their elevated circulating cortisol concentrations. Nonetheless, cycling female marmosets might in fact experience some of the pathophysiological consequences frequently associated with chronic hypercortisolemia, including steroid diabetes, muscular atrophy, bone demineralization, peptic ulceration, hypertension, suppression of the inflammatory response, and immunosuppression (9,43). If so, the increase in HPA activity that accompanies social dominance and reproductive cyclicity would

represent a cost of dominance in this species, offsetting, to some extent, the distinct reproductive benefits accruing to dominant females.

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