General and Comparative Endocrinology 113, 80–86 (1999)
Article ID gcen.1998.7183, available online at http://www.idealibrary.com on IDEAL®

Fecal Glucocorticoids Document Stress in Female Barbary Macaques (*Macaca sylvanus*)

Bernard Wallner,*[,1] Erich Möstl,† John Dittami,* and Hermann Prossinger‡

**Institute of Zoology, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria; †Institute of Biochemistry, University of Vienna, Josef Baumannstrasse 1, A-1221 Vienna, Austria; and ‡Institute of Human Biology, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria*

Accepted September 10, 1998

Patterns of received aggression and the endocrine response were related to an increase in fecal glucocorticoid metabolites in an intact semifree-ranging group of Barbary macaque females in order to quantify the social stress incurred over a 20-week observation period. The novel result showed that immunoreactive cortisol and 11-oxoetiocholanolone found in the feces can indeed determine the endocrine response of the adrenal gland after a social stressor. After HPLC separation of pooled fecal samples, EIA analyses using three different assays (corticosterone, cortisol, and 11-oxoetiocholanolone) to quantify immunoreactive steroids showed that the corticosterone EIA had no distinctive immunoreactive peaks. Cortisol and 11-oxoetiocholanolone immunoassays showed respectively four and two immunoreactive substances. Time series analyses revealed a behaviorally initiated increase in concentrations of cortisol and 11-oxoetiocholanolone equivalents. Furthermore, both hormone curves exhibit comparable time functions. Either antibody is very suitable for determining glucocorticoid secretion after periods of stress.

Aggression is a common response when members of group-living species attempt to monopolize resources. While purposes of aggression vary, they show uniform physiological stress reactions among affected individuals, and a physiological cascade of reactions to social hierarchies increases adrenal activity (Sapolsky, 1995). In addition to competition for access to ecological resources, sexual competition is a major motivation in the drive to dominate other members of the group (Dunbar, 1988). The metabolic effects of prolonged increases in cortisol levels lead to serious consequences for the organism (Thun and Schwarz, 1994): the general acute adaptation system of the organism fails and the stress response becomes destructive, producing a range of dysfunctions. Chronic stress suppresses the immune system (Peristein *et al.,* 1993) and changes metabolic (Jayo *et al.,* 1993) and gastrointestinal functions (Monnikes *et al.,* 1994). In social-living primates the effects of prolonged activation of the hypothalamo-pituitary-adrenal axis on reproductive potency of individuals are well documented (Sapolsky and Krey, 1988). Stress invoked by harassment or overt aggression represents an efficient proximate strategy for suppressing reproduction in subordinate individuals (Dunbar, 1989); in females, ovarian function is inhibited and spontaneous abortion occurs (Wasser and Starling, 1988).

Plasma cortisol concentrations are the most reliable markers of endocrine responses to social stress. Difficulties in collecting plasma samples and the effects of the collection procedure itself, especially under free or semifree conditions, have lead investigators to seek noninvasive approaches. The procedures for analyzing various steroids in urine and gonadal steroids in feces

[1] To whom correspondence should be addressed at Institute of Zoology, Althanstrasse 14, 1090 Vienna, Austria. Fax: +43-1-31336-778. E-mail: a8731gct@vm.univie.ac.at.

0016-6480/99 $30.00

are well established in primates (Ziegler *et al.*, 1996a,b; Whitten and Russell, 1996; Stavisky *et al.*, 1995). In most animals, steroids are excreted as inactive metabolites via urine or feces (Döcke, 1994). In feces, progesterone metabolites are the dominating substances (Schwarzenber *et al.*, 1996). The measurements of cortisol or cortisol metabolites in primate feces are currently unresolved problems in behavioral endocrinology. In cats, fecal cortisol metabolites are mainly present in polar, nonhydrolyzable forms (Graham and Brown, 1996); in ruminants, neither cortisol nor corticosterone was found in the feces; while in sheep, remnants of infused cortisol are excreted as 11,17-dioxoandrogens (Palme and Möstl, 1997).

The present study investigates patterns of received aggression within an intact group of female Barbary macaques (*Macaca sylvanus*) as a measurement of social stress. The behavioral results of this study represent a by-product of a project dealing with the size of perineal swelling in females in relation to their social status and endocrine condition (Wallner and Dittami, 1997). Ethological field studies describe the time course of the agonistic behavior and showed that the stress response can be detected in feces by determining fecal glucocorticoid metabolites. Enzyme immunoassays (EIA) were used to determine the quantities of immunoreactive corticosterone, cortisol, and 11-oxoetiocholanolone.

METHODS

Data Collection in the Field

This study was carried out during the nonreproductive season on a group of semifree-ranging Barbary macaques in Affenberg Salem (Germany). The group consisted of 45 adult females and 38 adult males. The behavior of 16 females was observed in the spring of 1995 in a 1 ha large area during a 20-week period; behavioral data were collected by 20-min focal sampling and were coded with the one/zero method (Altmann, 1974). The reception of intrasexual aggression was defined as a stress parameter to assess stress reactions. Aggression parameters partially followed Scott's and Deag's definition (Scott, 1974; Deag, 1974) and included threats, chases, and attacks with physical contact (grasping, hitting, and biting).

Fecal Sample Collection and Chemical Analysis

A total of 210 fecal samples was collected in which glucocorticoid metabolites were determined. The feces were collected once a week from the ground between 13:00 and 15:00 directly after excretion. Afternoon samples were preferred because then the feces are not mixed with urine; the collected feces were immediately frozen at −20°C.

The steroids for the enzyme immunoassay were extracted from 0.5 g feces with 1.5 ml water and 3 ml methanol. The antibodies for the corticosterone, cortisol, and 11-oxoetiocholanolone EIA were raised in rabbits. The characteristics and the cross-reactions of these three EIAs have been described elsewhere (Palme and Möstl, 1997).

For high pressure liquid chromatography (HPLC) separation, 0.5 g of pooled fecal samples was extracted with methanol (4 ml, 80%) and the supernatants were evaporated under nitrogen. The residue was reconstituted with a 0.5 ml water/$NaHCO_3$ solution (5%) and reextracted with diethylether (5 ml). The extracts were introduced into a Lichrosorb 10 µm Si60 25 × 0.4-cm^2 column (from Forschungszentrum Seibersdorf, Austria) and separated by HPLC using a mixture of *n*-hexane:chloroform:methanol (60:40:1; v:v; linear methanol gradient up to 6%; flow rate 2 ml/min). Within 36 min 107 fractions were collected. After separation, the organic solvents were evaporated and the residues redissolved in an assay buffer. The concentrations of immunoreactive materials were measured in each fraction using immunoassays for corticosterone, cortisol, and 11-oxoetiocholanolone metabolites. For pooled fecal samples, the intraassay coefficient of variation (CV) for cortisol was 9.4%, for 11-oxoetiocholanolone 11.4% ($n = 35$); while the interassay CV was 12.4 and 15.3% ($n = 12$), respectively.

Statistical Analyses

Weekly received aggression and hormone data were averaged for the different categories and means are reported with the standard deviation from the mean (SD). Comparisons between the categories were made using one-way ANOVA with the post hoc Bonferroni test. The interhormone metabolite was computed with the Wilcoxon matched-pairs test. Cross-correlation co-

efficients between single values and the averages of weekly steroid hormones and between received aggression and the hormonal data were estimated with time series analyses.

RESULTS

The cortisol immunoassay showed that four immunoreactive substances were eluted from the column. The cross-reacting steroids (fractions 45–82) were less polar than [^{3}H]cortisol, which was eluated in fraction 83. This showed that, although the pure eluated cortisol level was low in Barbary feces (<5 pg/sample; Fig. 1a), immunoreactive metabolites with different elution patterns (other than [^{3}H]cortisol) are present in the samples. Cortisol was only detectable in trace amounts; all subsequent values are expressed as cortisol equivalents. The 11-oxoetiocholanolone assay showed two immunoreactive peaks which resemble monohydroxylated androgens (Fig. 1b); the values are therefore expressed as 11-oxoetiocholanolone equivalents (11,17-dioxoandrogens). Moreover, the corticosterone EIA did not show immunoreactive peaks.

In Fig. 2 the curves from Individual 7 show both the weekly received aggressions and the excreted fecal glucocorticoids. These individual time series represent a correspondence between the observed behavior and the measured adrenal output (climaxing at Week 10). Furthermore, the strength of the hormonal response seemed to be related to the extent of received aggression. For her, it is evident that each of the two peaks in received aggression is followed in the subsequent weeks by elevated glucocorticoid concentrations. The time series analysis confirms this interpretation (Fig. 3): relative to received aggression, the cortisol equivalent has a lag of 1 to 2 weeks at the 95% confidence level, while for the 11-oxoetiocholanolone equivalent it is 0 to 2 weeks at the 95% confidence level.

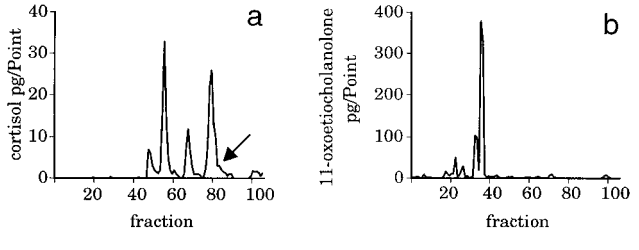

FIG. 1. HPLC separation of (a) immunoreactive amounts of cortisol and (b) 11-oxoetiocholanolone equivalent steroids. Each fraction was analyzed with EIAs for cortisol and 11-oxoetiocholanolone. (The arrow indicates the elution maximum of [^{3}H]cortisol.)

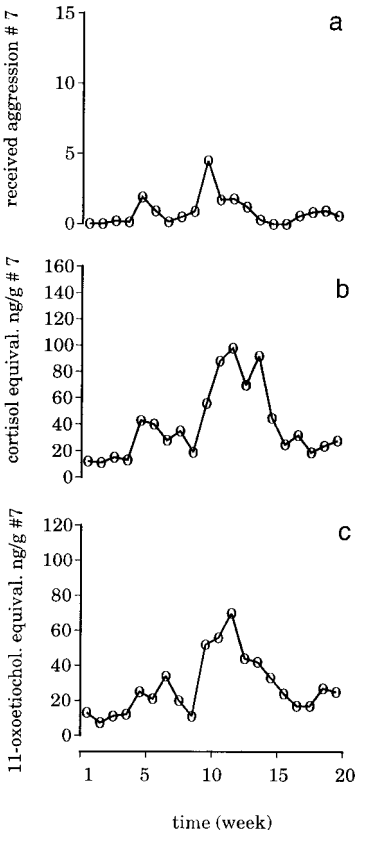

FIG. 2. Individual 7's time series of (a) received aggression and (b) excreted fecal glucocorticoids analyzed using cortisol and (c) 11-oxoetiocholanolone antibodies.

The received aggression averages varied significantly during the 20 weeks, being highest in Week 10 (Fig. 4a). Although the cause of the maximum was unclear, one particularly dominant female appeared to have a key role in its initiation. In that week, many aggressive encounters between this female and members of another family took place; thereupon perceived relatives and allies led to increased encounters. Along with the behavioral data, there were increased levels of immunoreactive adrenal substances in the feces (Figs. 4b and 4c), but their time course differed. The fecal

concentrations remained elevated for up to 4 weeks after the aggression had escalated. For 2 weeks after this behavioral stress, the focus animals showed the highest cortisol equivalent concentrations in feces (Fig. 4b). In comparison, the 11-oxoetiocholanolone equivalent levels were characterized by a more marked reaction in Week 12 (Fig. 4c). The cross-correlation factor (ccf) of time series analyses showed a time lag of 1 week between this received aggression and elevated cortisol equivalent concentrations in feces at the 95% confidence level; the time lag for the 11-oxoetiocholanolone equivalent was 2 weeks at higher than 95% confidence level (Figs. 5a and 5b). The ccf of cortisol equivalents and 11-oxoetiocholanolone equivalents peaked together (time lag 0 weeks at better then 95% confidence level; Fig. 5c).

It is concluded that if one steroid is a particularly weak signal then the other can be used as an adequate substitute. Furthermore, no statistical differences between the medians of cortisol equivalents and 11-oxoetiocholanolone equivalents concentrations could be found (Wilcoxon matched-pairs test; $P = 0.087$).

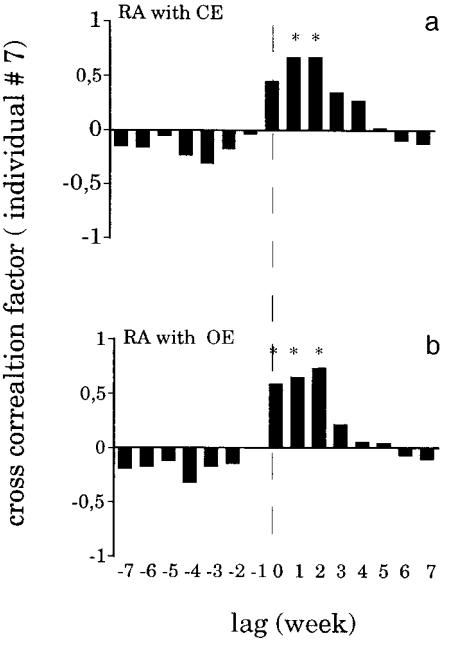

FIG. 3. Time series cross-correlations between (a) received aggression/cortisol equivalent titers and (b) received aggression/11-oxoetiocholanolone equivalent titers from Individual 7. (*statistically significant at 95% confidence level; RA, received aggression; CE, cortisol equivalent; OE, 11-oxoetiocholanolone equivalent.)

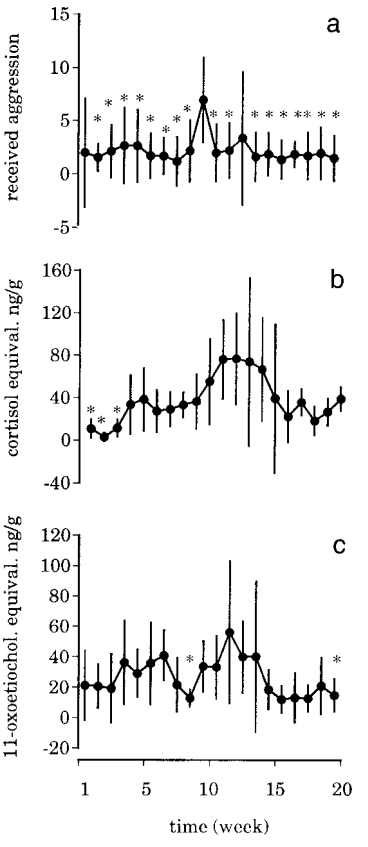

FIG. 4. Mean temporal progression of (a) received and excreted fecal glucocorticoids analyzed using (b) cortisol and (c) 11-oxoetiocholanolone antibodies. (Values are means ± SD; one-way ANOVA; test $df = 19$; $P < 0.05$; modified LSD (Bonferroni) test; *statistically significant at 95% confidence level (a) to Week 10, (b) to Week 11 and Week 12, (c) to Week 12.)

DISCUSSION

The antibodies used were directed against the three CMO derivates of the haptens. The cross-reactions showed that these antibodies recognize various steroids differeing in ring A of the molecule (Palme and Möstl, 1997). Therefore, all the assays can be considered as group specific. From the cross-reactions of the antibodies for cortisol and 11-oxoetiocholanolone, the measured metabolites are assumed to be derived from glucocorticoids. The presence of 11-oxoetiocholanolone in the feces of Barbary macaques agrees with fecal sheep data, where a 11-oxoetiocholanolone assay worked very well, but the cortisol EIA did not (Palme and Möstl, 1997). The 11-oxoetiocholanolone immuno-

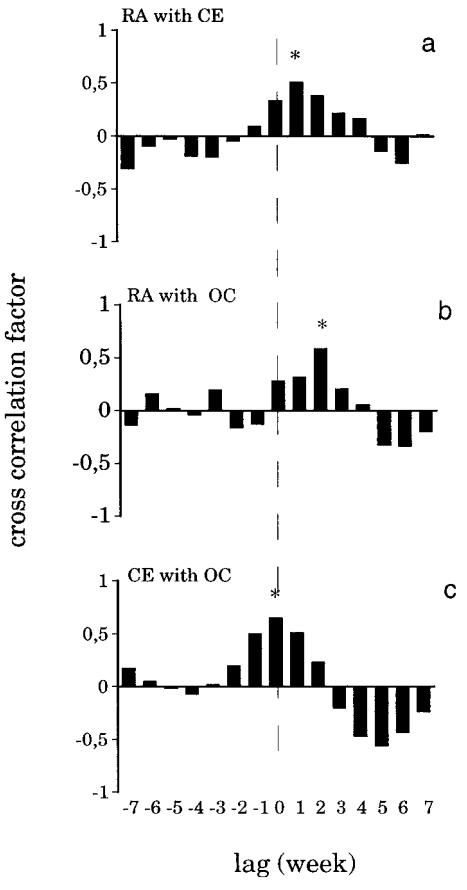

FIG. 5. Time series cross-correlations (a) received aggression / cortisol equivalent titers, (b) received aggression / 11-oxoetiocholanolone equivalent titers, and (c) cortisol equivalent / 11-oxoetiocholanolone equivalent concentrations. (*Statistically significant at 95% confidence level; RA, received aggression; CE, cortisol equivalent; OE, 11-oxoetiocholanolone equivalent.)

reactive metabolite did not originate from gonadal androgens because they have an oxo group at the C-11 position. Whitten *et al.* (1998) described cortisol as the main metabolite measured in the feces of chimpanzee; this contrasts with our findings, where cortisol was present only in trace amounts.

Time series analysis hints at a straightforward interpretation of the data, which is more conclusive when applied to the averages. At the 95% confidence level, the lag between received aggression and cortisol equivalent is 1 week, and for 11-oxoetiocholanolone concentrations 2 weeks. These detected lags point to a possibly different metabolic output mechanism between the two glucocorticoid metabolites. Further evidence is supplied by a direct delay analysis of the two glucocorticoids: the overlap implies their equivalence as a marker for received aggression, but the spread awaits a metabolic interpretation. The time series analysis documents that the adrenal glucocorticoid response is effected by received aggression.

From Week 11 (Figs. 3b and 3c) the fecal levels of cortisol equivalents and from Week 12 the 11-oxoetiocholanolone equivalent levels remain elevated up to Week 14; during this period the standard deviations are also larger. The perception of, and the reaction to, aggression may depend on the personality of the females (Kirschbaum *et al.*, 1995); perhaps this dependence can explain why some individuals show high and others low adreno-glucocorticoid responses to received aggression. In contrast to the hormonal data, received aggression values decrease markedly after Week 10. It may be that the physical stressor was then followed by psychological excitement, which manifests itself in elevated glucocorticoid concentrations. Psychobiological effects and their consequences are well known from winner / loser experiments, e.g., guinea pigs (Sachser and Lick, 1989), as well as from social interactions among marmoset monkeys (Abbott *et al.*, 1997) and among squirrel monkeys in the absence of social support (Levine *et al.*, 1997).

The activity along the hypothalamus-pituitary-adrenal axis may reflect a psychological component of a stressor (Stratakis and Chrousos, 1995; Levine, 1993), which is expressed in two ways. Physiologically, it causes an immediate hormonal reaction, which can be predicted by the *fight and flight* model, where stress hormones like epinephrine and cortisol play a major role (Christensen and Jensen, 1995). Emotionally, it represents a psychosocial dimension, which can lead to a prolonged, elevated discharge of cortisol caused by proximities to other individuals, even without their overt aggression. The intensity of this latter phenomenon is indicated by a 4-week increase in titers of cortisol equivalents, although aggressive interactions were relatively low. Analytically, such models would result in a slow, gradual decay curve of the glucocorticoids; one could describe this effect as a psychological half-life. It may be that the behavioral events in Week 10 produced an unstable and difficult-to-predict social circumstance. The group members were highly sensitized to any incident during this time period (Konarska *et al.*, 1990).

The present investigation provides evidence that the specifity of the antibody used in the analyses is crucial for determining glucocorticoid metabolites in the feces of macaques. Aggressive interactions produce prolonged physiological changes in individuals, which can be monitored in the excreted steroids.

ACKNOWLEDGMENTS

We thank Dr. W. Angst, the head of Affenberg Salem, whose generous and cooperative nature made this work possible. Dr. A. Paul, who supported the idea from the beginning, and Dr. J. Küster for her valuable information during the work.

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