Zoo Biology 11:165–176 (1992)

Urinary Monitoring of Adrenal Responses to Psychological Stressors in Domestic and Nondomestic Felids

Kathy Carlstead, Janine L. Brown, Steven L. Monfort, Richard Killens, and David E. Wildt

National Zoological Park, Smithsonian Institution, Washington, DC (K.C., J.L.B., S.L.M., D.E.W.); National Institutes of Health Animal Center, National Center for Research Resources, Bethesda, Maryland (R.K.)

The potential of assessing adrenal responses to psychological stressors through the radioimmunoassay of free cortisol in urine was examined in the domestic cat (*Felis catus*) and in three nondomestic felid species (*Felis geoffroyi, Felis bengalensis,* and *Felis concolor*). To determine the approximate clearance rate of an acute increase in glucocorticoid secretion, serial plasma and bladder urine samples were collected from eight domestic cats after a 0.125 mg adrenocorticotropic hormone (ACTH) challenge. Within 30 min of administration, mean serum cortisol concentrations increased tenfold. Urinary cortisol concentrations increased twofold by 2 hr post-ACTH and were correlated with the serum responses. Also, 16 domestic cats were anesthetized, injected with 0.125 mg ACTH, and serially bled for 3 hr. All urine was collected for 24 hr post-ACTH. Urinary cortisol concentrations were significantly elevated compared to pretreatment concentrations and were correlated to the serum cortisol response (net area under the response curve). In another experiment, urine was collected daily for a 7-day baseline period from 16 domestic cats housed in standard laboratory cages. Subsequently, 8 cats were subjected to 8 consecutive days of "stress," consisting of relocation, physical restraint, and jugular venipuncture. The other 8 cats were neither moved, nor handled, nor bled for the same period of time. Two patterns of response were observed among the "stressed" cats: urinary cortisol concentrations either increased or decreased between baseline and treatment periods. These response profiles differed from those of controls, which remained basal and unchanged over time. A fourth experiment involved relocating a female Geoffroy's cat, 4 leopard cats, and 2 pumas to a novel environment for 8–10 days. Urinary cortisol concentrations rose on the first day of relocation and remained

Received for publication September 13, 1990; revision accepted January 9, 1992.

Address reprint requests to Dr. Kathy Carlstead, National Zoological Park, Smithsonian Institution, Washington, DC 20008.

elevated above baseline for 5–7 days. Overall, these data suggest that adrenal responsiveness to psychological stressors in these four felid species can be assessed noninvasively by measuring coritsol in 24-hr urine samples. This research strategy may be useful for optimizing captive habitats to improve overall animal welfare and/or reproductive performance.

Key words: psychogenic stress, ACTH, cortisol, glucocorticoids, stress

INTRODUCTION

It is widely speculated that psychogenic stress can compromise health and suppress reproduction in many wildlife species. The establishment and maintenance of captive populations of small and medium-sized felids has been hampered by poor reproductive results that may be due, in part, to suboptimal housing/husbandry conditions [Mellen, 1991]. Little attention has been directed at assessing the nature or impact of environmental factors causing stress in felids.

The mammalian hypothalamic-pituitary-adrenal (HPA) axis is sensitive to environmental perturbations perceived as threatening or challenging. Acute adrenal responses to environmental change are typically assessed by measuring temporal profiles in pituitary adrenocorticotropic hormone (ACTH) and/or adrenal (glucocorticoid) hormones in the peripheral circulation [Baldwin and Stephens, 1973; Bassett and Cairncross, 1973; Hennessy and Levine, 1979; Dantzer et al., 1980; Flaherty et al., 1986]. However, this approach may be unsatisfactory for assessing adrenal activity in response to more chronically occurring stressors, such as caging conditions or husbandry routines. Hormone secretion is a dynamic process, and any given blood sample provides only a single "point in time" estimate. Stress-induced changes in the secretory pattern of corticosteroids usually involve increased frequency or amplitude of pulsatile episodes, often during only certain phases of the normal diurnal or ultradian variation but not others [Ladeweg, 1987]. In lieu of frequent blood sampling through catheterization (an impractical method for freely moving wild animals), monitoring fluctuations in urinary cortisol is considered a reliable means of estimating total changes in adrenal output pooled over time [Baum et al., 1974; Lasley, 1986; Ladeweg, 1987]. Increased urinary corticosteroids in response to psychological stressors have been measured in humans [Hamburg, 1962; Ursin and Murison, 1983; Bassett et al., 1987], domestic sheep [Berman et al., 1980], bighorn sheep [Miller, 1988], and captive rhesus monkeys [Mason et al., 1957, 1968].

A prerequisite to the routine use of urinary hormone analysis for tracking psychophysiological response is the validation of assay procedures. The objectives of this study were to 1) determine the validity of a commercially available radioimmunoassay (RIA) kit for quantifying free cortisol in felid urine, 2) determine the approximate clearance time of acute elevations in glucocorticoid secretion into urine, 3) demonstrate that fluctuations in urinary cortisol excretion reflect environmentally induced changes in HPA axis activity, and 4) assess the ability of this technique to monitor adrenal activity in nontractable, captive felids. Because our previous studies have demonstrated the utility of domestic animals as models for nondomestic species [Wildt et al., 1983, 1986; Wildt, 1990], initial studies were conducted in the more tractable laboratory cat. Resulting methods then were applied to the Geoffroy's cat (*Felis geoffroyi*), leopard cat (*Felis bengalensis*), and puma (*Felis concolor*).

MATERIALS AND METHODS

Animals and Urine Collection

For experiments 1–3, 16 adult male and 8 adult female domestic cats were housed individually in stainless steel, laboratory cages (1 × 1 × 1 m) at the National Institutes of Health (NIH) Animal Center for at least 1 month before initiating the studies. Dry cat chow (Ralston Purina Co., St. Louis, MO) and water were provided ad libitum, and canned meat was fed once daily. A standardized 12-hr photoperiod consisting of combined natural and fluorescent lighting was maintained in the colony room. Each cage contained a 15-cm-wide shelf, water and food bowl, and a double-layered plastic litter pan. The top pan was punctured with 0.5-mm-diameter holes and contained 0.5 liter of nonabsorbant cat litter (A.J. Buck, Owings Mills, MD), which allowed urine, but not feces, to pass through to the lower pan. Twenty-four-hour urine samples were collected daily at 10:00 AM and stored at −20°C until assayed.

For experiment 4, all felids were housed on exhibit at the National Zoological Park, Washington, DC, with the exception of leopard cats that were maintained initially at the NIH Animal Center. An adult female Geoffrey's cat was housed with a male in an indoor exhibit (3 × 3 × 3 m) that was furnished with two nestboxes (50 × 30 × 20 cm), plants, several large branches, and two aluminum pans. On 8 different days the female was observed between 9:00 AM and 12:00 PM to urinate in one particular pan, and freshly voided urine was obtained on these occasions. Two adult male and two adult female leopard cats were housed singly in enclosures (3.5 × 1.5 × 2.6 m) at the NIH Animal Center. The cages contained a metal shelf; a hanging, wire cage; and an aluminum pan. Daily urine was collected with a syringe from the cage floor or from an aluminum pan on a daily basis. Two sibling female pumas were housed together in an outdoor, wire mesh enclosure covering 83 m^2 of earthen substrate planted with foliage and containing tree stumps and branches, a large rock, and a pond. A concrete den at the rear of the exhibit (1.5 × 1.7 × 1.5 m), where the animals were fed and sometimes slept, was the site used by both animals for depositing urine. A pooled sample from both individuals was collected with a syringe each morning from the den floor.

Experiment 1

Experiment 1 was conducted to determine the clearance rate of cortisol into urine following an acute increase in adrenocortical activity. Between 10:00 AM and 12:00 PM, 8 male domestic cats were induced into a light surgical plane of anesthesia using ketamine hydrochloride (HCl)/acepromazine maleate (10:1 ratio, 20.0 mg/kg and 2.0 mg/kg body weight, respectively) administered i.m. Anesthesia was maintained for 2 hr with supplemental ketamine HCl/acepromazine injections, while each cat was subjected to an ACTH challenge. After obtaining a pretreatment blood sample 20 min after anesthesia induction (time 0) 0.125 mg ACTH was administered i.m., and blood samples were collected at 15, 30, 45, 60, 90, 120, 150, and 180 min post-ACTH via a jugular catheter. A pre-ACTH urine sample was collected from the litter pan of each cat 2–4 hr before the ACTH challenge. During anesthesia, all urine was collected from the bladder at 2, 3, and 4 hr post-ACTH through a urinary catheter inserted 1 hr after anesthesia induction.

Experiment 2

To determine whether acute increases in adrenocortical activity result in elevated urinary cortisol, changes in serum cortisol responses to ACTH over a 4-hr period were compared and correlated to changes in 24-hr urinary cortisol concentrations. Eight male and 8 female domestic cats were lightly anesthetized with an injection of Telazol (5 mg/kg body weight; A.H. Robbins, Richmond, VA) between 9:00 and 10:00 AM, and an indwelling catheter was inserted into a cephalic vein. One hour later, a 2-ml blood sample was taken prior to an i.m. injection of 0.125 mg ACTH. Blood samples were subsequently drawn every 15 min for 1 hr, then every 30 min for an additional 3 hr. Urine was collected from the litter pans the morning of the day before, the day of, and the day after the procedure.

Experiment 3

Baseline urinary cortisol values were established by collecting urine for 7 consecutive days (week 1) from 12 male and 4 female cats under standard laboratory conditions. During a subsequent 8-day period (week 2), 8 of the cats were subjected to a daily "stress" regimen. Because individual response differences were expected, the baseline period allowed each individual to serve as its own control. Beginning at 10:30 AM, each "stress" cat was placed in a transport cage (58 × 40 × 46 cm) and then left in an unfamiliar part of the building for 30 min. Each animal then was placed in a Nylon canvas restraint bag, bled via jugular venipuncture (3 ml), and transferred to the "home" cage. After a 1-hr clotting period, blood was centrifuged (10 min, 1,800 *g*) and the serum stored at −20°C until assayed. The other eight cats served as nonstressed, nonhandled controls. Urine was collected daily from all 16 cats as described above.

Experiment 4

Morning urine was collected from one Geoffroy's cat, four leopard cats, and two pumas for 5–7 days before and 8–10 days after each was moved to an unfamiliar exhibit or holding cage in a novel facility. The female Geoffroy's cat was captured, placed in a transport box, translocated to another building, and housed alone for 8 days in a wire cage (1.5 × 2 × 2 m), which was provisioned with a nest box and an aluminum pan. Other animals (four golden lion tamarins, *Leontopithecus rosalia rosalia*) were in close visual proximity, and the cage was located at a site frequented by animal caretakers. Leopard cats were moved from the NIH Animal Center to a holding facility at the National Zoological Park and housed singly in 2.5 × 1.3 × 1.3 m cages containing a 60 × 30 × 40 cm nest box and aluminum urination pan. Pumas were moved to an indoor holding facility with a 19 m^2 concrete and wire mesh cage containing two wooden shelves for resting.

Cortisol Analyses

Cortisol was measured in urine and serum using a solid-phase, ^{125}I radioimmunoassay kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA). The antiserum was specific for free, unconjugated cortisol and had the following cross-reactivities: 1.4% with corticosterone; 1.0% with tetrahydrocortisol; and <1.0% with cortisone, 11-deoxycortisol, 21-deoxycortisone, estriol, estrone, progesterone, and tetrahydrocortisone. Urine (0.5 ml) was combined with methylene

chloride (1 ml) (Optima Grade; Fisher Chemical Co., Fair Lawn, NJ), shaken vigorously (10 min), and then centrifuged (1,500 *g* for 5 min). The lower methylene chloride phase was removed by aspiration with a pipet and placed in another polypropylene tube. Recovery of tritiated cortisol (2,500 cpm) added to urine following methylene chloride extraction was >90%. Two hundred microliters of extractant was dried down in antibody-coated tubes and assayed in duplicate. Twenty-five microliters of unextracted serum was assayed according to the kit protocol. Samples were counted for radioactivity in a gamma counter (TM Analytic model 1290) with a counting efficiency of 83%. Assay sensitivity was 2.0 ng/ml. Urinary cortisol values (ng/ml) were indexed by creatinine concentration (mg/ml CR) to compensate for changes in fluid balance [Lasley, 1986; Monfort et al., 1989].

High-Pressure Liquid Chromatography (HPLC)

To determine the relative contribution of immunoreactive cortisol, domestic cat urine was analyzed using HPLC separation methods described previously [Monfort et al., 1991]. Pooled urine (3 ml) was combined with 7,000 cpm ^{3}H-cortisol (100 μl) and 6 ml dichloromethane, vortexed (30 sec), and centrifuged for 12 min (1,500*g*). Three milliliters of dichloromethane (1.5 ml urine equivalent) was aspirated, dried down, reconstituted in 55 μl methanol, and injected onto a reverse-phase Microsorb column (RP C-18, 5-μm-diameter particle size). A linear gradient of 20–100% methanol in water within 80 min (1 ml/min flow rate, 1.0 ml fractions) was used to separate gluccocorticoid components. Separate portions of column eluates were collected directly for counting of tracer and assayed for immunoreactivity using the previously described cortisol radioimmunoassay (RIA).

Statistics

Average values for serum (ng/ml) and urinary cortisol (ng/mg creatinine) were calculated as mean ± standard error of the mean (SEM). In all experiments, changes in mean urinary cortisol concentrations between baseline and treatment conditions were tested for significance using dependent Student's t tests. Net increases in adrenal output were assessed by mathematical calculation of the area under the serum cortisol time-response curve above the time 0 level. Because individual response differences were expected, the baseline period of experiment 3 allowed each individual to serve as its own control. Responses to handling and blood sampling were analyzed using a multivariate profile analysis of variance model of repeated measures. To determine the degree of correspondence between single blood samples and 24-hr urine samples in characterizing adrenal activity, for each "stressed" cat, Pearson's correlation coefficients were calculated for serum and urinary cortisol values.

RESULTS

Validation of RIA for Urinary Cortisol

Recovery of unlabelled cortisol (5–300 ng) added to 0.5 ml domestic cat urine was 99.0% ($y = 0.98x + 0.41$; $r = 0.99$; $P < .001$). Inter- and intraassay coefficients of variation were 6.8% and 5.9%, respectively. Analysis of 25 μl of 1 ml of extractant from two pools of domestic cat urine and one pool each of Geoffrey's cat, leopard cat, and puma urine resulted in displacement curves parallel to the cortisol standard curve (Fig. 1).

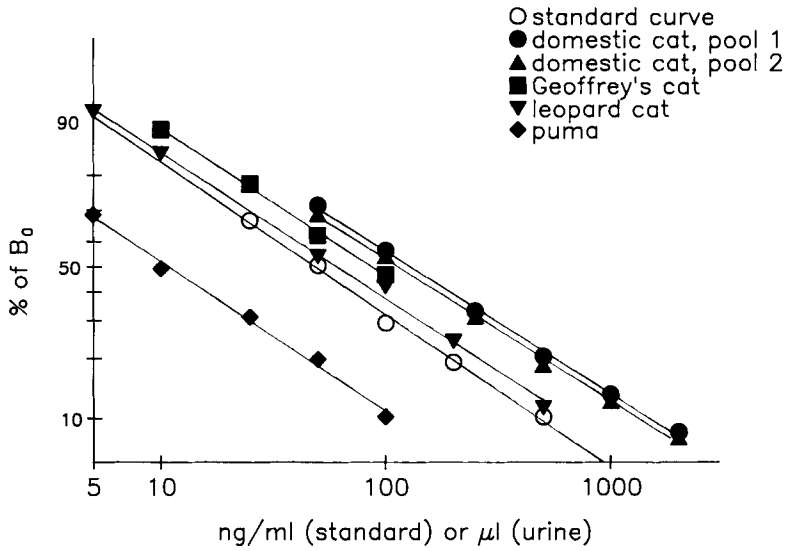

Fig. 1. Displacement curves for the cortisol standard and serial dilutions of two pools of domestic feline urine and one pool each of Geoffroy's cat, leopard cat, and puma urine in the radioimmunoassay for cortisol. B_0 = maximum binding.

The HPLC cochromatographic profile of cortisol immunoreactivity coeluted with ^{3}H-labelled cortisol. Recovery of cortisol in fractions 39–43 was 93.0%.

Experiment 1

Overall mean basal serum cortisol concentration at time 0 (pre-ACTH) was 4.6 ± 0.7 ng/ml. The ACTH bolus increased serum cortisol tenfold to a mean peak of 53.1 ± 9.3 ng/ml at 30 min (Fig. 2). Mean serum cortisol concentrations decreased to baseline within 2 hr but then rose again as each cat began to recover from anesthesia (Fig. 2). Basal cortisol concentration from morning urine collected from the litter pans was 4.2 ± 0.9 ng/mg CR pre-ACTH. Bladder urine was recovered from 7 of the 8 ACTH-treated cats. Concentrations were elevated significantly above basal levels at 2 hr (9.9 ± 2.2 ng/mg CR, P = .023) post-ACTH but not at 3 or 4 hr post-ACTH. The net serum cortisol response from 0 to 60 min post-ACTH was significantly correlated to the urinary cortisol concentration of bladder urine 3 hr post-ACTH (n = 7, R = .886, P = .008) but not at 2 or 4 hr. Thus cortisol is cleared into urine between 2 and 3 hr post-ACTH.

Experiment 2

Basal serum cortisol concentration increased approximately twofold from 45.0 ± 7.1 ng/mg/CR prior to ACTH injection to a mean peak of 81.1 ± 7.6 ng/mg CR 45 min post-ACTH. Cortisol levels returned and remained at baseline by 90 min post-ACTH. Urine collected from litter pans 24 hr later contained cortisol concentrations (7.2 ± 1.1 ng/mg CR) that were elevated (P < .05) from those collected the day before (5.1 ± 0.5) and the day of (4.5 ± 0.7) the ACTH treatment. A significant

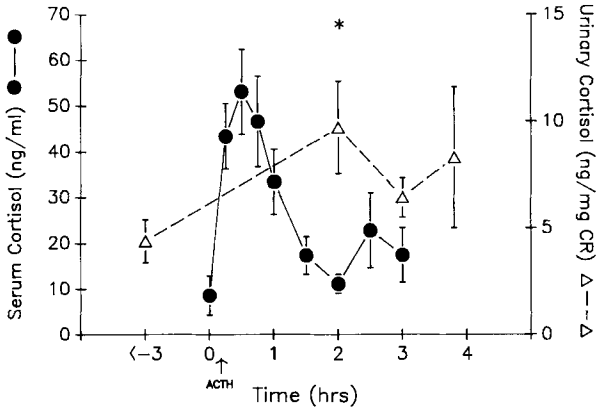

Fig. 2. Serum and urinary cortisol concentrations in domestic cats before and after administration of 0.125 mg ACTH. Cats were maintained under anesthesia for the first 2 hr. Post-ACTH urine samples were collected from the bladder via catheterization. Asterisk denotes significant differences from baseline urinary cortisol concentrations ($P < 0.05$).

correlation was found between the net area under the serum response curve from 0 to 240 min and urinary cortisol 24 hr later (n = 16, R = .558, $P < .05$).

Experiment 3

Results of the multivariate profile ANOVA did not indicate an overall effect of group ("stress" or control) on urinary cortisol concentrations for the 7 days of the baseline period (week 1) contrasted with the 8 days of the treatment (week 2) (df = 1,14, F = .966, $P = .342$). However, closer examination of the data for each individual cat indicated that one-half of the "stressed" animals responded to the week 2 procedure with mean urinary cortisol concentrations that, when tested individually with dependent t tests, were elevated ($P < .05$) above week 1 concentrations. The other one-half of the cats responded with decrements compared to prestress levels, three of which were significant ($P < .05$). In contrast, none of the control cats demonstrated any significant change in mean urinary cortisol concentrations between weeks 1 and 2. Therefore, the "stressed" cats could be divided into two groups, those exhibiting increases (n = 4) and those exhibiting decreases (n = 4) in mean urinary cortisol concentrations in response to handling and blood sampling (Fig. 3). Profile analysis revealed a significant overall effect of group on contrasts between weeks 1 and 2 (df = 2,13, F = 22.033, $P < .0001$); response patterns were not parallel between the "increasing" cats and the controls (df = 1,13, F = 27.417, $P < .0001$) and the "decreasing" cats and controls (df = 1,13, F = 4.417, $P = 0.056$).

For four of the "stressed" cats, significant positive correlations were found between serum cortisol concentrations at the time of blood sampling and cortisol concentration 24 hr later (cats A–D, Table 1). For one cat, a significant negative correlation was found (cat E). Three of the cats responding to the treatment with decreases in urinary cortisol were those demonstrating nonsignificant correlations (cats F–H).

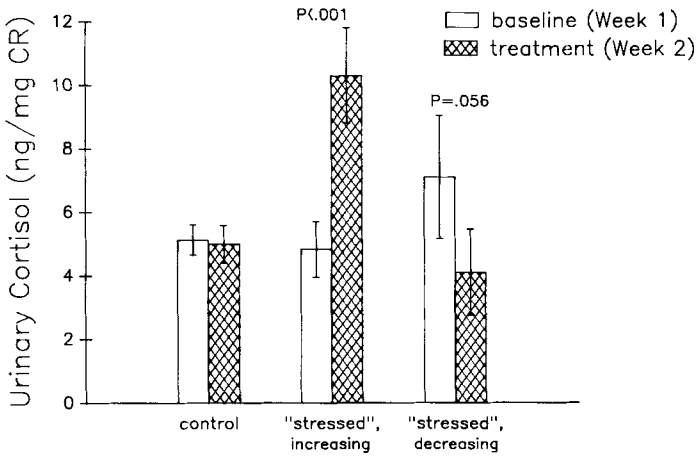

Fig. 3. Mean (±SEM) urinary cortisol concentrations in control and "stressed" cats during week 1 baseline (n = 7 days) and week 2 treatment periods (n = 8 days). "Stressed" cats could be categorized into two groups based on whether mean urinary cortisol concentrations increased (4 cats) or decreased (4 cats) between weeks of the study. *P* values indicate significant differences in the pattern of change compared to controls (8 cats).

TABLE 1. Correlations for individual cats between serum and urinary cortisol concentrations (n = 8 days)

Cat	Pearson's R	P	Direction of change in experiment 2
A	0.72	<0.05	Increase
B	0.74	<0.05	Increase
C	0.88	<0.05	Increase
D	0.69	<0.05	Decrease
E	−0.73	<0.05	Increase
F	0.57	NS	Decrease
G	0.31	NS	Decrease
H	0.35	NS	Decrease

Experiment 4

Urinary cortisol concentrations increased sevenfold (Geoffroy's cat), threefold (mean of four leopard cats), and 15-fold (pumas) within 1 day of removal to a new environment compared to the preceding baseline values (Fig. 4). Cortisol remained elevated for 5 days after the transfer in the Geoffroy's cat ($P < .05$), for 7 days in the leopard cats ($P = .056$), and for 5 days in the pumas ($P < .05$).

DISCUSSION

These results suggest that the analysis of urinary cortisol content can be used as a noninvasive method for assessing psychogenic changes in adrenal activity in felids. The RIA was validated by demonstrating 1) parallelism between urine displacement curves and the cortisol standard curve, 2) significant recovery of known amounts of cortisol added to domestic cat urine, 3) recovery of a single immunoreactive peak by

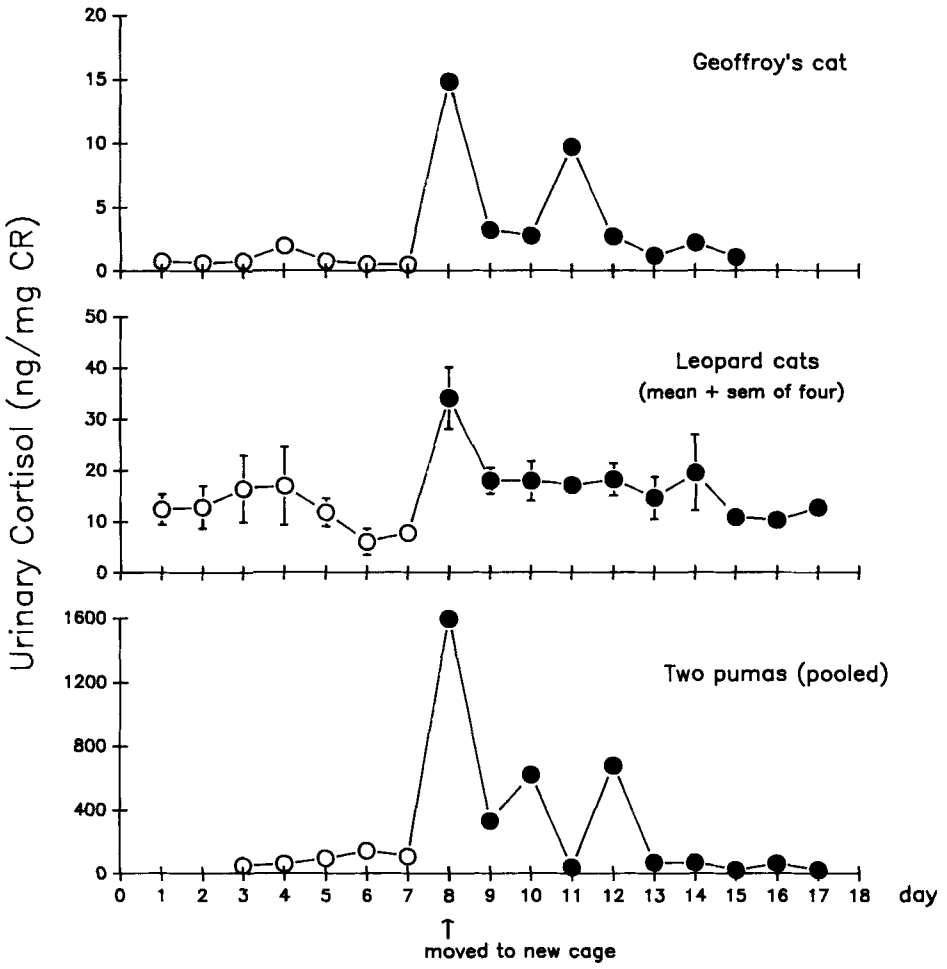

Fig. 4. Sequential urinary cortisol concentrations for a female Geoffroy's cat, the mean (±SEM) of four leopard cats, and from pooled urine samples of two female pumas collected in the home cage for 5–7 days (○) and after being moved to a novel cage for 8–10 days (●).

HPLC that coeluted with ^{3}H-cortisol, and 4) a relationship between changes in urinary cortisol and physiological and psychological factors known to affect adrenal activity. Results indicate that cortisol is cleared from the peripheral circulation in approximately 2 hr, similar to the time observed in bighorn sheep [Miller, 1988]. Furthermore, an experimentally induced increase in serum cortisol resulted in an increase in cortisol concentration in the subsequent 24-hr urine sample.

The equivocal results of experiment 3, however, underscore the importance of psychological processes in determining physiological responses to experimentally administered stressors. The "stressor" of handling and blood sampling in this experiment actually produced two, opposing response patterns: one-half of the "stressed" cats exhibited overall increases in urinary cortisol, whereas the other one-half exhibited decreases. Adrenocortical responses to exogenous stressors are

known to be influenced by prior experience with environmental challenges (including experience with people) in a variety of species, [rats, Sakellaris and Vernikos-Danellis, 1975; Vernikos et al., 1982; Uphouse et al., 1983; Armario et al., 1985; mice, Soblosky and Thurmond, 1986; sheep, Berman et al., 1980; pigs, Dantzer et al., 1980; Hemsworth et al., 1987]. For this reason, a baseline period was included in the experimental design so that each cat could serve as its own control. The stressor employed was psychologically complex; the cats could respond to the novelty of relocation in a carrying cage, to restraint, to handling by humans, to anticipation of blood sampling, and to the pain of the sampling procedure itself. The data suggest that aspects of these procedures were, indeed, aversive for some of the cats, as indicated by the significant increase in urinary cortisol. In contrast, those showing significant response decrements relative to the baseline period may have been responding to the rewarding properties of being held during the blood sampling. In rats, pituitary-adrenal activity has been shown to decrease when large rewards are obtained or expected or when reward conditions are improved over what was expected based on past experience [Levine and Coover, 1976; Coover, 1983]. The four cats exhibiting response decrements were, in retrospect, the most tractable and affiliative with people.

The response of nondomestic cats to the psychological stressor of relocation to a new cage was much more consistent, and a profound and rapid increase in urinary cortisol was observed in all cases. The more consistent response to this stressor may be due to its chronic nature (compared to the handling procedure used with the domestic cats) and to the unlikelihood of wildlife species finding relocation to be a rewarding experience.

Scientific assessment of the welfare status of confined animals is a growing area of research interest as ethical questions are raised about the psychological well-being of animals maintained for research, economic, education, and entertainment purposes. Likewise, diminishing populations of free-living animals are creating a critical need for captive breeding programs. These two trends emphasize the need to elucidate the adequacy of captive habitats for optimal behavior, health, and reproduction in wildlife species. The sources of psychogenic stress in laboratory and zoo-housed animals can be identified by studying the interrelationships between behavioral and physiological responsiveness to environmental conditions. The present study suggests that analysis of adrenocortical hormones in 24-hr urine samples is a valid and potentially valuable approach for studying stress sensitivity in the context of environmental, genetic, and developmental factors in felids.

CONCLUSIONS

1. A radioimmunoassay for assessing free cortisol in felid urine was validated by dose-response curves parallel to a known, standard curve; recovery of 99.0% of added, unlabelled cortisol in urine; and coelution of the HPLC profile of felid urinary cortisol with labelled cortisol.

2. An injection of ACTH and serial blood sampling caused 1) an increase in serum cortisol that was cleared into urine 2 hr later, and 2) a rise in urinary cortisol concentration in a subsequent 24-hr urine sample.

3. Psychological aspects of a blood sampling procedure had measurable, indi-

vidual-specific effects on urinary cortisol concentrations. These effects were stressful for some cats but not for others.

4. Relocation to a novel cage caused a 5–7-day increase in urinary cortisol concentration in three species of nondomestic felids.

ACKNOWLEDGMENTS

This research was supported by grants from the Geraldine R. Dodge Foundation, Friends of the National Zoo, the Women's Committee of the Smithsonian Associates, and the Scholarly Studies Program of the Smithsonian Institution. We greatly appreciate the assistance of animal caretakers Ray Keels and Kathleen James.

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