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A Non-Invasive Method for Assessing Adrenal Activity in the Chinchilla (*Chinchilla Lanigera*)

MARINA F. PONZIO[1, 2*], STEVEN L. MONFORT[2], JUAN M. BUSSO[1], VIVIANA G. DABBENE[3], RUBÉN D. RUIZ[1] AND MARTA FIOL DE CUNEO[1]
[1]*Instituto de Fisiología, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, X5000ESU, Argentina*
[2]*Conservation & Research Center, Smithsonian's National Zoological Park, Front Royal, Virginia 22630*
[3]*CEPROCOR, Colonia Santa María 5164, Santa María de Punilla, Córdoba, Argentina*

ABSTRACT The Chinchilla is a rodent that was once abundant in the central Andes of South America. Excessive hunting for fur greatly reduced its distribution at the beginning of the twentieth century, and today *Chinchilla* species are nearly extinct in the wild. Although protected, wild populations of chinchilla are still declining. In general, this species has received little research attention and its biology is poorly understood. Improvements in captive breeding, husbandry, and genetic management are needed to ensure the conservation of the species. In this study, a noninvasive corticosteroid hormone monitoring technique was validated for use in *Chinchilla lanigera*. Two male domestic chinchillas were administered ^{3}H-corticosterone (i.m.) to determine the time course and relative proportion of urinary and fecal steroid metabolites. Most radioactivity was detected in urine and feces 5–10 and ~30 h post-isotope administration, respectively. Corticosteroid immunoreactivity was assessed by corticosterone radioimmunoassay (RIA) and cortisol enzyme immunoassay (EIA). High-pressure liquid chromatography (HPLC) separation of corticosteroid metabolites in unprocessed urine revealed the presence of highly polar corticosteroid metabolites, but after enzymatic hydrolysis and diethyl ether extraction, most immunoreactivity co-eluted with unconjugated cortisol. A 'cause-and-effect' relationship between the administration of exogenous adrenocorticotrophic hormone (ACTH), and the appearance of increased urinary corticosteroid metabolites demonstrated the physiological relevance of these measures for evaluating adrenal status in male chinchillas. From a conservation perspective, these methods can aid in situ and ex situ initiatives designed to evaluate how environmental conditions and management strategies affect overall animal health, well-being and reproduction. *J. Exp. Zool. 301A:218–227, 2004.*

INTRODUCTION

The chinchilla, a strictly nocturnal rodent, is a member of the suborder Hystricomorpha, in which two distinct species are recognized: *Chinchilla lanigera* and *Chinchilla brevicaudata*. These animals produce the most valuable pelts in the world, and both were once abundant in the central Andes of South America. Excessive hunting for fur and habitat fragmentation greatly reduced the number and distribution of individuals at the beginning of the twentieth century. Wild populations were harvested over a prolonged period of time at a higher rate than reproduction and recruitment rates could sustain (i.e., chinchillas produce two to three offspring per litter, once or twice each year) (Grau, '86; Jiménez, '95, '96; Weir, '96).

Consequences for the wild populations were soon evident (Jiménez, '94); today, *Chinchilla* spp. are almost extinct in the wild and they are listed on Appendix I of CITES (CITES, '73). Although protected, the number of individuals is still declining but the reasons are poorly understood. It is evident that without active management, research, and conservation, wild chinchilla

*Correspondence to: Instituto de Fisiología, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Santa Rosa 1085, X5000ESU, Córdoba, Argentina. E-mail: mponzio@mater.fcm.unc.edu.ar.

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populations will almost certainly become extinct in the near future (Jiménez, '94).

Although native chinchilla are extremely rare, a hybrid produced by cross-breeding the two chinchilla taxa has been domesticated, bred, and selected for superior fur production for more than 80 years. Thus, the domestic chinchilla is different from both of the wild species (Grau, '86). Today the chinchilla represents a peculiar wildlife paradox: no other furbearer is so common in captivity yet so rare in the wild (Rice, '88).

Research conducted in common nondomestic or domestic animal models can be extremely useful for developing an improved understanding of the biology of their endangered counterparts (Wildt et al., '86; Carlstead et al., '92; Graham and Brown, '96). Similarly, knowledge obtained from studies of farmed chinchilla is likely to be directly applicable to their wild counterparts, and may be important for enhancing captive breeding efforts designed to provide a hedge against extinction.

It is well-known that the "stress" associated with sub-optimal housing/husbandry conditions can compromise animal health and well-being, and adversely impact reproductive function in many wild and domestic species (Sapolsky, '85; Mellen, '91; Rivier and Rivest, '91; Dobson and Smith, '95). Breeders of the domestic chinchilla have commonly observed fur-plucking and inter-sexual aggression (Vanjonack and Johnson, '73; Grau, '86; Tisljar et al., 2002), behaviors that have been attributed to "captivity stress" in other species (Wielebnowski, 2002).

Physiological measures of the stress response have typically relied upon the evaluation of serum or plasma glucocorticoids. However, attempts to obtain repeated blood samples from chinchilla by either venipuncture or chronic indwelling catheterization were unsuccessful, in part, because of small vein size and their stress-susceptible nature (unpublished observation). Very small amounts of blood were obtained by other authors through orbital or peripheral venipuncture or tail tip laceration (Brookhyser et al., '77; Grozmadzka-Ostrowska and Zalewska, '84; Grozmadzka-Ostrowska et al., '85; Tappa et al., '89).

Fortunately, noninvasive fecal and urinary corticosteroid monitoring can now be used to assess adrenal status in nondomestic species (Brown et al., '95; Jurke et al., '97; Monfort et al., '98; Terio et al., '99; Monfort, 2003). Noninvasive approaches in the chinchilla could permit long-term endocrine monitoring while avoiding the potentially stress-evoking stimuli of restraint and translocation, as well as risks associated with repeated venipuncture, including vascular damage, infection, and anemia. Noninvasive corticoid monitoring could be particularly useful for investigating the relationship between various husbandry/management strategies and physiological stress in the chinchilla. An improved understanding of these relationships may help animal managers to develop more effective captive breeding programs for both domestic and wild chinchillas.

The overall objective of this study was to demonstrate the validity of noninvasive corticosteroid monitoring for evaluating adrenal responsiveness in the chinchilla. Studies were designed to assess the technical capability for accurately quantifying corticosteroids in chinchilla excreta, as well as to prove the "physiological validity" of these noninvasive measurements. Technical validation was demonstrated by 1) determining the time-course of radiolabeled urinary and fecal steroid metabolites excretion after ^{3}H-corticosterone administration, 2) investigating the putative identity and relative proportion of the urinary and fecal corticosteroids metabolites, and 3) demonstrating specificity, sensitivity, accuracy, and precision of a corticosterone RIA and a cortisol EIA for quantifying excreted corticosteroid metabolites. Physiological validity was established by demonstrating a cause-and-effect relationship between the activation of the adrenal gland through the administration of exogenous adrenocorticotrophic hormone (ACTH), and the corresponding excretion of immunoreactive urinary corticosteroid metabolites.

MATERIALS AND METHODS

Animals and sample collection

Adult domestic *Chinchilla lanigera* males (500–600 g weight) were individually-housed in stainless steel cages, fed a pelleted chinchilla mixture (Cargill SACI, Pilar, Córdoba, Argentina) and water ad libitum. Animals were maintained in an indoor laboratory facility with exposure to natural fluctuations in photoperiod and temperature. The design here employed has been approved by our animal care and use committee, and conforms to NIH guidelines.

Separation of urine and feces at the time of excretion was possible by making slight modifications to a regular housing cage. Cage bottoms were triple-layered steel litter pans. The top pan had transversal steel rods that provided a supportive

surface for the animal. The middle pan consisted of steel mesh (1.0 mm diameter openings) that permitted urine, but not feces, to pass through to the lower pan, which forms a steel funnel that directed urine into a collection tube. All samples were stored without preservative (−20°C) until processing. To permit habituation to the new environment, animals were moved into their metabolic cages for at least 10 d before the onset of experiments.

Radiolabeled corticosterone administration

To determine the time-course of corticosterone metabolite excretion, and the proportion of metabolites excreted in urine versus feces, two males were given a bolus injection (i.m.) of ~5 μCi ^{3}H-corticosterone (NEN Life Science Products, Inc., Boston, MA), plus 250 μg unlabeled corticosterone (Sigma Chemical Co., St. Louis, MO) in 0.3 ml of PBS (pH 7). To estimate the total quantity of radioactivity administered, 50 μl was removed from the syringe immediately before injection for radioactive counting. After isotope administration, syringes were rinsed with ethanol and the residual radioactivity was counted and subtracted from the total amount administered.

For the steroid metabolism trials, urine and fecal samples were collected at ~4–h intervals for two days before and four days after isotope administration. To determine total radioactivity, excreted aliquots of each sample (0.05 g feces, 0.05 ml urine) were counted for radioactivity and the values were multiplied by the total fecal mass or urine volume at each time point. Urine samples were counted directly in 3 ml of scintillation fluid, whereas fecal samples were first homogenized in 0.5 ml ethanol and then mixed with 16 ml scintillation fluid, to minimize sample quench. The relative excretory fate of ^{3}H-corticosterone (i.e., urine versus feces) was determined by dividing the radioactivity detected in urine or feces by the total quantity of label.

The relative proportion of water soluble (WS, i.e., polar metabolites) versus ether soluble (ES, i.e., nonpolar metabolites) ^{3}H-corticosterone metabolites in urine or fecal extracts was determined by radioactive counting after diethyl ether extraction (1:10 aqueous:ether, vol:vol) (Monfort et al., '98).

High pressure liquid chromatography (HPLC)

The quantity and relative distribution of corticosteroid metabolites in urine and fecal extracts were determined after reverse-phase HPLC (Graham and Brown, 1996; Monfort et al., '90, '97). Before HPLC, urine (1 ml) or fecal extract (reconstituted in 1 ml acetic acid, pH 5.5) were pre-filtered through a C–18 matrix column (Bakerbond SPE 7020–03, Phillipsburg, NJ, USA) and eluted with 5 ml of 80% methanol to remove contaminants (Heikkinen et al., '81).

Recovery of known amounts of radioactivity after cartridge filtration was 92.8%. Filtered extracts were evaporated to dryness and reconstituted in 300 μl of methanol. Fecal, but not urinary, extracts were also re-filtered through a 45–μm filter. A 55–μl portion of each fluid was then loaded onto the HPLC and eluted over 80 min (flow rate, 1 ml/min) with a gradient consisting of 20–80% methanol in water. To assist the characterization of the separated metabolites, samples contained tritiated steroids (^{3}H-cortisol, ^{3}H-corticosterone, and ^{3}H-desoxicorticosterone) added before HPLC to serve as co-chromatographic markers. Radioactivity and/or corticosteroid immunoreactivity were determined in separate aliquots of each eluate.

To determine the identity of the major immunoreactivity found in urine, 1 ml of pooled urine was pre-filtered as described above; the filtrate was then dried and reconstituted in acetic acid (1 ml, pH 5.5) and extracted with diethyl ether. Residual aqueous extractants were enzymatically-hydrolyzed with β-glucuronidase/aryl sulfatase (2,000 Fishman U glucuronidase activity, 16,000 Roy U sulfatase activity at pH 5.5) for 16 h (37°C), followed by a second ether extraction to separate WS from ES forms. To ensure that conjugated and unconjugated steroid forms were completely separated in the first diethyl ether extraction and before hydrolysis, the WS form obtained was subjected to HPLC; the relatively polar fractions (1–30) obtained were then pooled and subjected to enzymatic hydrolysis. HPLC was applied to each resulting form and the eluates obtained were evaluated as described above.

Immunoassay procedures

Fecal and urine sample processing

Fecal samples were extracted as previously described (Wasser et al., '94). Briefly, dried,

pulverized feces (0.025 g) were combined with 10 ml of 90% ethanol (in water) and boiled (20 min) in a heated water bath (90–95°C). After centrifugation (500 g, 15 min), the supernatant was evaporated under a stream of air and resuspended in 1 ml of methanol. Fecal extraction efficiency, based on recovery of ^{3}H-corticosterone added before extraction, was 96.8±1.2% (n=20). Unprocessed urine samples were diluted (1:50 to 1:500) in the appropriate assay buffer, and to account for day-to-day fluctuations in fluid balance, hormone concentrations were expressed as hormone mass per mg creatinine (Cr) (Taussky, '54; Monfort et al., '90).

Radioimmunoassay (RIA)

Corticosterone metabolites were evaluated in urine and HPLC eluates using a ^{125}I-Corticosterone RIA kit (ICN Biomedicals Inc., Costa Mesa, CA). The manufacturer reports that the antiserum cross-reacts with desoxicorticosterone (0.34%), testosterone (0.10%), cortisol (0.05%), aldosterone (0.03%), progesterone (0.02%), androstenedione (0.01%), 5α-dihydrotestosterone (0.01%), and <0.01 % with all other steroids tested. Parallel displacement curves were obtained by comparing serial dilutions of pooled chinchilla urine and fecal extracts with standard hormone preparations (urine and feces, r^2=0.98 and 0.94 for the standard and diluted pool, respectively). Urine samples were diluted 1:500 with steroid diluent (provided with the kit). Recovery of known amounts of corticosterone added to a diluted pool of urine was 90.4±13.7% (y=18.08+0.79x; r^2=0.93). Inter-assay coefficients of variation (CV) for two separate internal controls were 7.8 and 11.9% (n=5). Intra-assay CV was <5% and assay sensitivity was 12.5 ng/ml.

Enzyme immunoassay (EIA)

Urine samples and HPLC fractions were analyzed for cortisol metabolites by EIA as described previously (Munro et al., '91), with minor modifications (Bellem et al., '95). Horseradish peroxidase ligands and polyclonal antisera (cortisol-R4866) were provided by C. Munro (University of California, Davis, CA). The antiserum crossreacts with prednisolone (9.9%), prednisone (6.3%), cortisone (5.0%), corticosterone (0.7%), 21-deoxycortisone (0.5%), deoxycorticosterone (0.3%), progesterone (0.2%), 11–desoxycortisol (0.2%), 17α-hydroxyprogesterone (0.2%), and <0.1 % with all other steroids tested. Parallel displacement curves were obtained by comparing serial dilutions of pooled chinchilla urine and fecal extracts with standard hormone preparations (urine, r^2=0.97 and 0.96 for the standard and diluted pool, respectively; feces, r^2=0.98 and 0.97 for the standard and diluted pool, respectively). Urine samples were diluted 1:500 and samples were assayed in duplicate. Recovery of known amounts of cortisol added to a pool of diluted urine was 86.3±3.7% (y=2.12−0.84x, r^2=0.99). Inter-assay CVs for two separate internal controls were 13.4 and 15.3% (n=15). Intra-assay CV was <10% and assay sensitivity was 0.078 ng/ml.

ACTH challenge

To determine the feasibility of detecting acute increases in adrenocortical activity via excreted corticosteroid metabolites, two adult males were injected (i.m.) once with 2 IU/kg of gel ACTH (Acthelea gel, Elea Laboratories, Buenos Aires, Argentina). For each individual, pre-treatment urine samples served as "controls" for comparison with post-ACTH adrenal responsiveness. Urine was collected at approximately 4–h intervals, for 2 d before and 4 d after ACTH administration.

Statistical analysis

Basic descriptive statistics were applied to the data (Statistica software package, CSS: StatSoft Inc.) and the results were expressed as Mean± Standard Error of the Mean (SEM), except those corresponding to hormonal concentrations from all the HPLC results.

RESULTS

A total of 45.5±11.3% (n=2) ^{3}H-corticosterone was recovered in urine and feces within 82 h of isotope administration; of this, 86.9±0.07% of metabolized radiolabel was excreted into urine whereas only 13.1±0.1% in feces (Table 1). After isotope administration, peak radioactive metabolite excretion occurred ~5–10 h and ~30 h later in urine and feces, respectively (Fig. 1). Differential ether extraction of peak-excretion samples revealed a high proportion of polar (i.e., >90% WS forms) urinary ^{3}H -corticosterone metabolites, whereas the majority of the fecal metabolites were nonpolar (ES forms, Table 1).

The vast majority (>85%, Table 1) of corticosteroids were excreted in urine, so fecal steroid metabolites were not evaluated by HPLC. Chromatographic separation of unprocessed chinchilla urine revealed at least four immunoreactive

'corticosterone' (i.e., eluates were assessed using a corticosterone RIA) metabolites (Fig. 2a); the first three polar metabolites eluted near the solvent front (fraction 3–20), whereas a broad-based increase in corticosterone immunoreactivity eluted (fractions 41–48) between cortisol and corticosterone reference standards. Simultaneous assessments of the same eluates using a cortisol EIA revealed the presence of several polar immunoreactive peaks (Fig. 2b, fractions 3–15), and another immunoreactive peak that clearly co-eluted with the cortisol standard (fractions 36–43). Comparison of the two immunoreactive profiles revealed that the cortisol EIA (maximum peak, 600 ng/ml) detected more immunoreactivity than the corticosterone RIA (maximum peak, 82 ng/ml).

TABLE 1. Excretory fate of injected ^{3}H-Corticosterone in Chinchilla laniger

	% Total radioactivity excreted	% Water soluble forms	% Ether soluble forms
Urine	86.9±0.07	90.5±5.7	9.5±5.7
Feces	13.1±0.08	20.8±14.3	79.2±14.3

The values are expressed as mean±SEM.

After ether extraction, >90% of urinary corticosteroid metabolites remained in the aqueous phase (i.e., WS forms). Although both immunoassays detected corticosteroid metabolites in the ES fraction (HPLC data not shown) that co-eluted with unconjugated corticosterone (0.32 ng/ml) and/or cortisol (70.7 ng/ml), the relative contribution of these nonpolar (ES forms) metabolites was a small proportion of all corticosteroid immunoreactivity detected in urine.

HPLC analysis of the WS fraction that had been subjected to enzymatic hydrolysis and subsequent diethyl ether extraction revealed that the majority (>75%) of polar corticosteroid metabolites were hydrolysable steroid conjugates of corticosterone (Fig. 3a) and cortisol (Fig. 3b). However, the cortisol EIA detected 25–fold more immunoreactivity (~800 ng/ml cortisol) compared to the corticosterone RIA (~30 ng/ml corticosterone). A small proportion (<25% of total immunoreactivity)

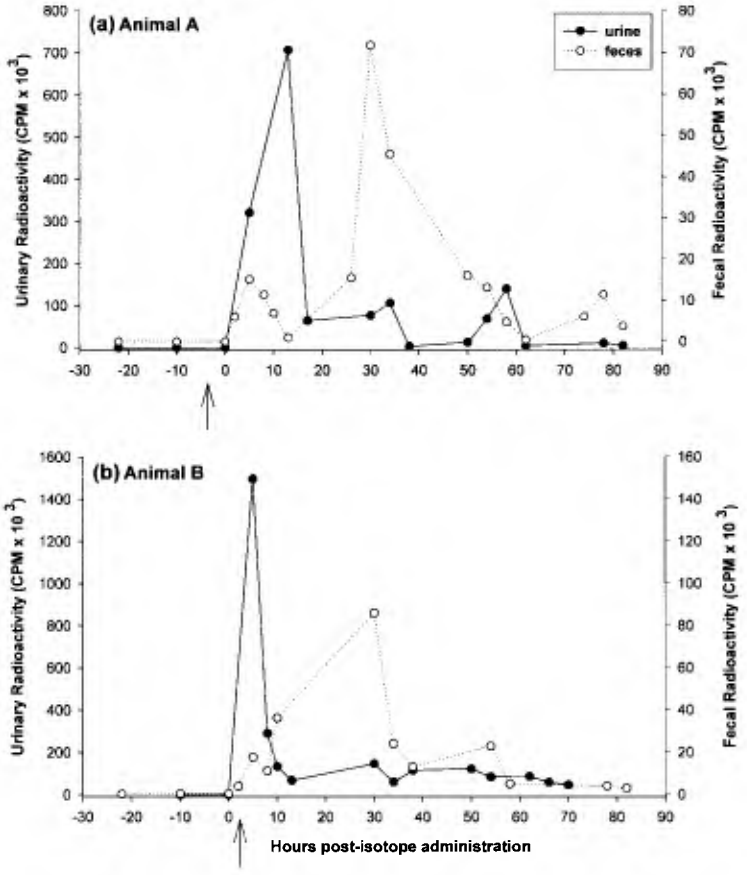

Fig. 1. Time-course of ^{3}H-cortisone excretion into urine and feces in two male domestic chinchillas (animals A and B). The radioisotope was injected (i.m.) at 0 time (arrow).

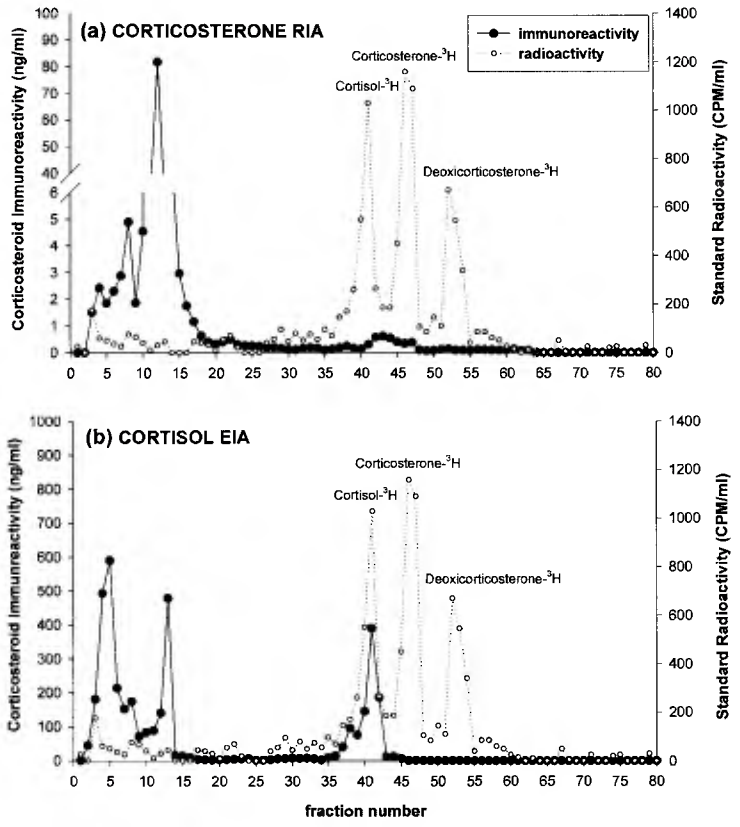

Fig. 2. HPLC separation of urinary corticosteroid metabolites in the male domestic chinchilla. Tritiated cortisol, corticosterone, and deoxicorticosterone were added as reference tracers. Immunoreactivity of each fraction was determined by corticosterone RIA (panel a) and cortisol EIA (panel b).

of immunoreactivity remained in the residual hydrolyzed-extracted urine.

For ACTH challenge experiments, urinary corticosterone immunoreactivity peaked (~4–fold above baseline) 5–10 h post-ACTH administration in both males (closed circles), with peak concentrations of 57.4 and 250.7 ng/mg Cr for animals C (Fig. 4a) and D (Fig. 4b), respectively. Temporal excretion patterns in urinary cortisol (open circles) were similar, and peak cortisol immunoreactivity was elevated ~7–fold higher than baseline concentrations. Despite temporal similarities in excretion patterns, peak cortisol immunoreactivity (Animal C, 3985.9 μg/mg Cr; Animal D, 5863.9 μg/mg Cr) was more than 3,000–fold greater (note different mass units) than corticosterone immunoreactivity (Animal C, 57.4 ng/mg Cr; Animal D, 250.7 ng/mg Cr).

DISCUSSION

The route of excretion (proportion excreted in urine vs. feces), the excretion lag-time (time from appearance in blood circulation to excretion in urine/feces), and metabolic form of excreted glucocorticoids differ between species (Monfort, 2003). Therefore, the aim of our study was to obtain basic knowledge about the metabolism and excretion of immunoreactive urinary and fecal corticosteroids in the chinchilla. This information is an essential prerequisite for developing a valid method for noninvasively assessing adrenal activity in this species. This was the first study designed to examine adrenal steroid excretion as an index of adrenal function in *Chinchilla lanigera*.

Bolus injection of radiolabeled steroid, differential extraction and subsequent HPLC analysis revealed that the majority (>85%) of corticosterone metabolites were excreted in urine, after an excretion lag-time of approximately 5–10 h. The vast majority of immunoreactive corticosteroids were excreted as conjugated forms of cortisol, and to a much lesser extent, of corticosterone.

Physiological validity was demonstrated by establishing a 'cause-and-effect' relationship

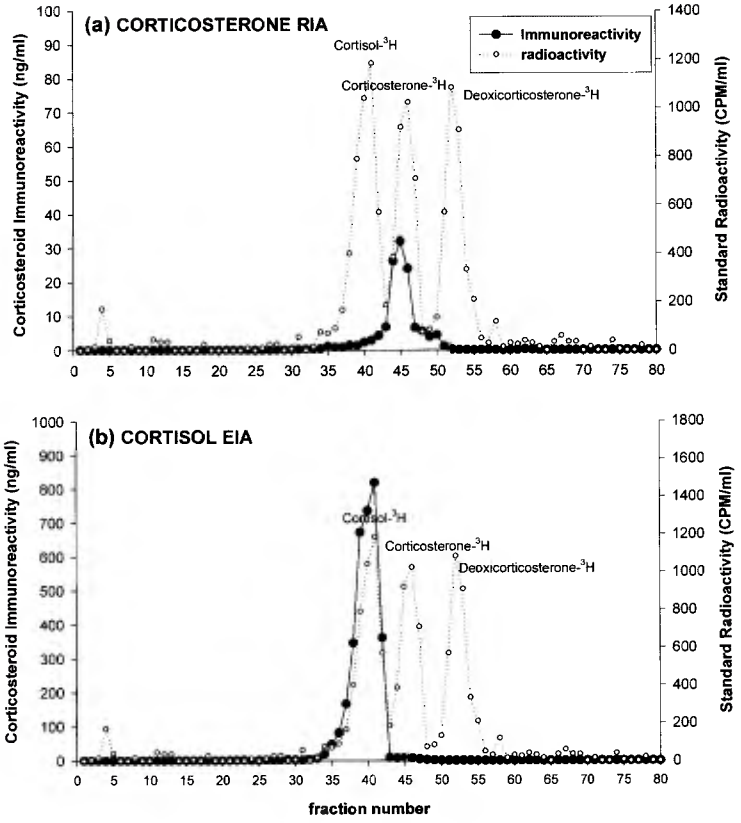

Fig. 3. HPLC separation of enzymatically hydrolysable urinary corticosteroid metabolites in male domestic chinchilla urine. Tritiated cortisol, corticosterone, and deoxicorticosterone were added as reference tracers. Immunoreactivity of each fraction was determined by corticosterone RIA (panel a) and cortisol EIA (panel b).

between the administration of exogenous ACTH, and the subsequent excretion of urinary corticosteroid metabolites. Overall, these results confirmed that urinary corticosteroid metabolites provided a valid and feasible measure to noninvasively monitor changes in adrenocortical activity that was superior to fecal corticosteroid measures.

In most species, circulating glucocorticosteroids are generally bound to plasma carrier proteins, which limits the excretion of urinary free cortisol and/or corticosterone. However, in some species tetra-hydro-glucuronide corticosteroid derivatives are produced by hepatic metabolism. These freely soluble corticosteroid conjugates are unbound in the blood circulation and are rapidly excreted in the urine, in part by tubular secretion (Axelrod and Reisine, '84; Munck and Guyre, '86). Although more work is needed for confirmation, the finding that chinchilla excrete large quantities of readily hydrolysable corticosteroids in urine suggests that a similar metabolic pathway may also exist in *Chinchilla lanigera*.

Cortisol (or its conjugates), were the predominant corticosteroid forms excreted after adrenal activation in the chinchilla. Despite the finding that urinary cortisol metabolites were excreted in much greater quantities than corticosterone metabolites, both measures were useful for tracking a temporal increase in adrenal activity after the administration of exogenous ACTH. Nevertheless, increased immunoreactivity detected using the cortisol EIA suggests that this immunoassay is probably a more appropriate tool for noninvasively assessing adrenal activity in the chinchilla. It is clear, however, that whichever method (i.e., cortisol EIA or corticosterone RIA) is employed, it is important to carefully characterize 'baseline' excretory patterns to account for individual-animal variation.

The chinchilla has been severely overexploited by humans, and the native species are on the brink of extinction (Jiménez, '94). However, at present only a few studies have focused on its reproductive physiology (Weir, '96; Ponce et al., '98a, b; Carrascosa et al., 2001; Bekyurek

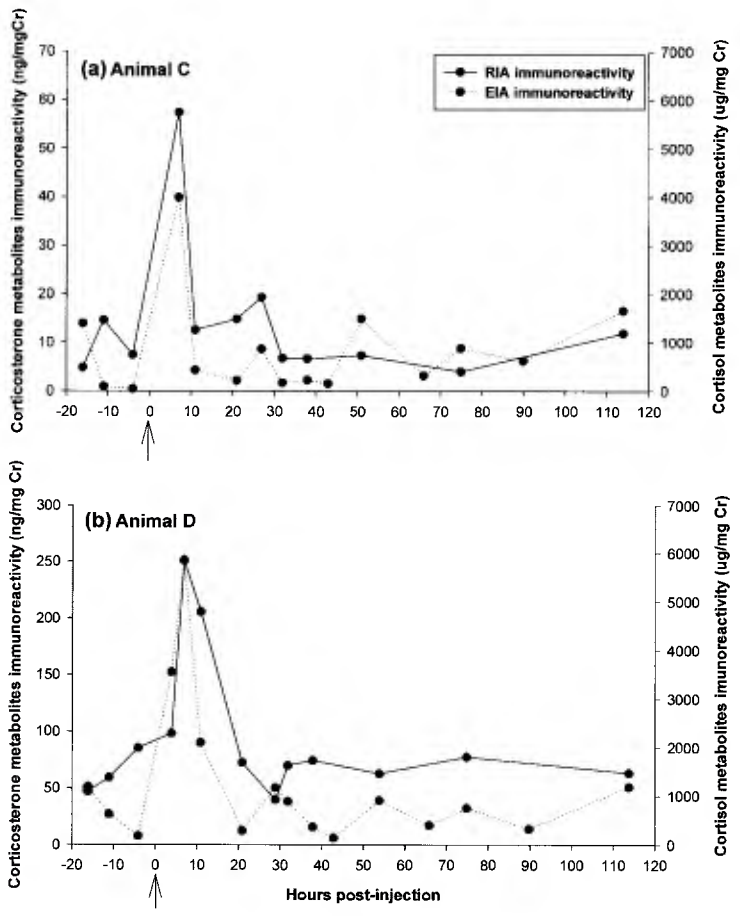

Fig. 4. Urinary corticosteroid immunoreactivity in two male domestic chinchillas (animals C and D), before and after an injection (i.m.) of 2 IU/kg of gel ACTH at time 0 (see arrow). Immunoreactivity of each sample was determined by corticosterone RIA and cortisol EIA.

et al., 2002), and no studies have examined the interrelationships between animal well-being, stress and reproductive fitness. More work is necessary to determine whether objective examinations of adrenal corticosteroid excretion are useful for evaluating the impact of 'stress' on chinchilla reproductive fitness. The availability of the method validated in the present study will improve our understanding of stress physiology in the chinchilla and this, in turn, may aid in developing new management strategies that will help ensure the future survival of the wild populations.

CONCLUSIONS

Based on our results, we conclude that:

Urine is the principal excretion route for corticosteroid metabolites in chinchilla.

HPLC analysis reveals that the majority of corticosteroids in chinchilla are excreted as readily hydrolysable steroid conjugates of cortisol.

Adrenal responsiveness to exogenous ACTH confirmed the physiological validity of urinary corticosteroid monitoring for evaluating adrenal activity.

Urinary corticosteroid measures have tremendous potential for evaluating the impact of stress on reproductive fitness in the chinchilla.

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