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Assessment of urine and fecal testosterone metabolite excretion in *Chinchilla lanigera* males

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Abstract

Endemic chinchilla (*Chinchilla* spp.) populations are nearly extinct in the wild (South America). In captive animals (*Chinchilla lanigera* and *C. brevicaudata*), reproduction is characterized by poor fertility and limited by seasonal breeding patterns. Techniques applied for studying male reproductive physiology in these species are often invasive and stressful (i.e. repeated blood sampling for sexual steroids analysis). To evaluate endocrine testicular function, the present experiments were designed to (a) determine the main route of testosterone excretion (¹⁴C-testosterone infusion in four males); (b) validate urine and fecal testosterone metabolite measurements (HPLC was used to separate metabolites and immunoreactivity was assessed in all metabolites using a commercial testosterone radioimmunoassay, and parallelism, accuracy and precision tests were conducted to validate the immunoassay); and (c) investigate the biological relevance of the techniques applied (quantification of testosterone metabolite excretion into urine and feces from five males injected with hCG and comparison between 10 males and 10 females). Radiolabelled metabolites of ¹⁴C-testosterone were excreted, 84.7 ± 4.2 % in urine and 15.2 ± 3.9 % in feces. A total of 82.7 ± 4.2% of urinary and 45.7 ± 13.6% of fecal radioactivity was excreted over the first 24 h period post-infusion (metabolite concentration peaked at 8.2 ± 2.5 h and 22.0 ± 7.0 h, respectively). Several urinary and fecal androgen metabolites were separated by HPLC but only fecal metabolites were associated with native testosterone;

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however, there was immunoreactivity in more than one metabolite derived from ^{14}C -testosterone. After hCG administration, an increase in androgen metabolite excretion was observed ($p < 0.05$). Males excreted greater amounts daily of urinary androgen metabolites as compared with females ($p < 0.05$); this difference was not evident in feces. Results of the present study indicate that the procedure used is a reliable and non-invasive method to repeatedly monitor variations in testicular endocrine activity in this species. It can be a useful tool that would help ensure the survival of the wild populations as well as to provide the basis for a more efficient use by the fur industry.

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1. Introduction

Most of the knowledge about endocrine modulation of reproductive physiology in *Chinchilla* spp. has been derived from studies in females (Gromadzka-Ostrowska and Zalewska, 1984; Gromadzka-Osrtowska et al., 1985). With respect to male reproduction, recently obtained results described the functional activity of epididymal or electroejaculated spermatozoa in the chinchilla (Ponce et al. 1998a, 1998b; Carrascosa et al., 2001), but at present there is no available information about sex steroid hormones.

Conventional methods for studying gonadal activity usually rely on invasive procedures, such as the analysis of serially collected blood samples (Wildt et al., 1995; Schwarzenberger et al., 1996). Repeated blood sampling for steroid analysis is usually impractical for small rodents, as handling itself can alter hormone concentrations and large blood quantities may also be required (Muir et al., 2001; Touma et al., 2003). Non-invasive methods, such as measuring gonadal hormone metabolites excreted into urine or feces, are attractive alternatives for studying some aspects related to reproductive physiology. Advances in this area are relevant for the application of assisted reproductive techniques in valuable domestic or endangered wild species (Palme et al., 1996; Holt and Pickard, 1999; Monfort, 2003).

Chinchilla spp. are categorized as threatened (CITES I) (Amori and Gippoliti, 2001) in a six-species family (*Chinchillidae*) from western and southern South America (Bronson, 1999). At present, the presence of remaining colonies in the Argentine Andes is uncertain (Redford and Wisenberg, 1992; Chebez, 1992). Besides, few studies have been performed in the wild strain of the species (Jiménez, 1995, 1996; Cortés et al., 2002).

Domesticated *Chinchilla* spp. still share some genomic characteristics with their counterparts in the wild (Zuleta et al., 2001); captive animals have poor fertility and reproduction is limited by seasonal breeding patterns (Weir, 1970; Gromadzka et al., 1985), and at present, the application of the modern reproductive techniques is limited by the limited knowledge about the reproductive physiology of this species. Due to the limited information studying wild and domesticated chinchillas, information that relates to reproductive physiology in domesticated animals may aid in developing new management strategies that would help ensure the survival of the wild populations, as well as in providing for a more efficient use by the fur industry.

We have previously determined that the main route of corticosteroid metabolite excretion is the urine (Ponzio et al., 2004); therefore, we hypothesize that excreted testosterone metabolites could follow a similar excretory route.

Applying a non-invasive technique in male *Chinchilla lanigera*, a study was designed to: (1) investigate the time course, number, proportion and percentages excreted into urine or feces of exogenous ^{14}C -testosterone excreted metabolites; (2) validate a radioimmunoassay for the androgen metabolites of greatest percentage that are excreted into urine and feces and (3) evaluate responses to exogenous hormonal treatment (hCG) and sexual differences in immunoreactive testosterone metabolite excretion.

2. Materials and methods

2.1. Animals

A total of 29 domesticated chinchillas (*C. lanigera*) were utilized in the present study. They were housed individually in stainless steel cages (0.32 m in width \times 0.32 m in height \times 0.49 m in depth), maintained indoors and exposed to natural photoperiod in Córdoba (Argentina) with a temperature range of 17–26 °C, with food (Rodent chow, Cargill) and water ad libitum. The body weight of the animals was: 575.34 \pm 17.19 g (males; $n = 19$) and 578.29 \pm 16.70 g (females; $n = 10$).

The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NHI, publication 85–23, revised 1996).

2.2. Radiolabelled infusion

To determine the time course and the proportion of testosterone metabolite excretion into urine and feces, four intact males were treated (i.m.) with 0.75 μCi ^{14}C -testosterone (40 $\mu\text{Ci}/\text{mL}$; Life Science Products Inc.) plus 200 μg of unlabelled testosterone (Schering AG) in a total volume of 400 μL isotonic solution. To determine total radioactivity (Wallac optiphase “hiphase” 2 scintillation fluid, Fisher Chemicals), urine and fecal samples were individually collected at 8 h intervals before isotope injection and at 4 h intervals for 7 days after treatment from pans with non-absorbent plastic strips. After isotope administration, syringes were rinsed with ethanol and the residual radioactivity was counted and subtracted from the total pre-injection. All samples were frozen at -20°C until processing. Then, fecal samples were individually solubilized by boiling in 1 mL of ethanol and the entire homogenate was dissolved in 16 mL of scintillation fluid and diluted in four aliquots of the same volume for counting. Aliquots of individually thawed samples (0.5 mL of urine, 0.5 g feces) were counted and all values were multiplied by total urine volumes and fecal weights to determine the total radioactivity excreted (Brown et al. 1996).

2.3. Feces and urine samples processing

All urine samples (except those that were radiolabelled) were collected in tubes containing 0.5 mL of ethanol as preservative according to Brown et al. (1995), and then were

centrifuged for 15 min (400 g); the supernatant and collected fecal samples were frozen at -20°C until processing. Fecal sample extraction was performed according to Monfort et al. (1993). Briefly, all samples (0.18–0.2 g) were lyophilized and pulverized (except samples from radioinfusion); then, they were boiled in absolute ethanol (20 min) and centrifuged (20 min, 400 g). The supernatant was completely dried in a heated water bath under air, the tubes were rinsed twice with ethanol, evaporated to dryness, and reconstituted in 1 mL of methanol; before radioimmunoassay, urinary and fecal extracts were further diluted in PBS (see below).

The proportion of water-soluble (conjugated) or ether-soluble (unconjugated) metabolites from peak radioactive samples was determined in unprocessed urine (1 mL) and in fecal extracts; after reconstitution in methanol, fecal extracts were taken to dryness, resuspended in 1 mL of PBS. Urinary and fecal steroids were extracted with nine volumes of diethyl ether (Brown et al., 1994). Because unconjugated metabolites were negligible, only the conjugated ones were processed by HPLC.

Processing efficiency of urinary and fecal endogenously metabolized ^{14}C -testosterone was determined by quantifying such metabolites in fecal extracts (0.2 g) and in unprocessed urine samples (50 μL) showing peak radioactivity. To assess the recovery of exogenous steroids, ^3H -hydrocorticosterone (Life Science Products Inc.; 12,500 dpm/tube) was added to urine and fecal samples before steroid extractions (Brown et al., 1996; Monfort et al., 1997).

2.4. HPLC methodology

From peak-radiolabelled samples, the number and relative proportions of testosterone metabolites in unprocessed urine and fecal extracts were determined by reverse-phase HPLC (Sphevedone ODS 2–250 \times 4.6 mm, 5 μm column, Rheodyne 7125 injector, Water Alliance 2690 equipment). Samples containing water-soluble metabolites were evaporated to dryness under air, resuspended in 200 μL of methanol (Fisher Chemical), and stored at 4°C . Twenty-four hours later, samples were sonicated (Ultrasonic bath Cole Parmer 8892) for 15 min while warming at room temperature and then, 500 μL of PBS were added and sonicated again for 10 min. Steroids were extracted using a solid-phase technique described by Dodds et al. (1995). In brief, samples were passed through a C-18 matrix column (Superclean LC-18 PK/54-Supelco, Bakerbond SPE C₁₈) and eluted with 5 mL of methanol (80 %). After filtration, samples were evaporated to dryness under air and resuspended in 200 μL of methanol (HPLC grade) and the elution by HPLC was performed using the same methanol: water gradient (20–100 % : 80–0 %) over 120 min (Dodds et al., 1995; Brown et al., 1996). To assess radioactivity and immunoreactivity, all HPLC fractions were taken to dryness and reconstituted in PBS.

2.5. Testosterone radioimmunoassay

Immunoreactivity in unprocessed urine samples (assay dilution 1:4 in PBS), in fecal extracts (assay dilution 1:8 in PBS) and in HPLC fractions were measured using a ^{125}I -testosterone RIA (Total Testosterone, DPC, Coat-A-Count); the antiserum for testosterone has less than 5% cross-reactivity to other steroids, except 19-nortestosterone (20%) in

serum, plasma or urine (data provided by the company). The kit is equipped with human serum-based calibrators. The intra-assay coefficients of variation (CV) for the greater and lesser concentration urine control samples (see below) were $3.7 \pm 0.8\%$ and $5.0 \pm 2.8\%$, respectively, while the CV for the internal control of kit was $5.4 \pm 1.6\%$. The inter-assay coefficients of variation (CV) for the greater and lesser concentration urine control samples were 8.4% and 13.1%, respectively, while the CV for the internal control of the kit was 8.5% ($n = 5$ each group). The following tests were performed to validate the assay:

- (1) *Parallelism*: The standard curve was generated from calibrators and a regression line fit; a representative pooled urine sample ($n = 16$ animals) was serially diluted to generate another regression curve. The testosterone assay was also validated for pooled fecal extracts ($n = 20$). This test was performed to determine whether urine and fecal extracts dilutions behave immunologically in a similar manner to the testosterone calibrators.
- (2) *Accuracy*: The recovery curve was generated by adding steroid calibrators ($n = 3$ for each point) in increasing amounts (10–800 ng/dL) to urine samples or fecal extracts (least concentration steroid samples were used according to parallelism tests, assay dilutions 1:32 PBS for urine and 1:64 PBS for feces). The recovered amounts include a percentage of added calibrator plus the amount contained in the original sample. Regression analysis was performed to determine the relationship between x (testosterone added) and y (testosterone recovered).
- (3) *Precision*: This characteristic was assessed by calculation of intra- and inter-assay coefficients of variation in the hormonal measurements performed in controls ($n = 2$) provided with the kit and two of each pooled urine and fecal extracts. They were diluted to measure at 20 and 80% binding (the lesser and upper ends of the sensitivity range of the urine and fecal extract curves from parallelism) (Biddlecombe and Law, 1996; Monfort, 2003).

Immunoreactivity in analyzed samples was expressed as follows: in unprocessed urine, androgen concentrations on a per day basis and, as well as, hormone mass per mg creatinine (in order to account for day-to-day fluctuations in fluid balance) (Monfort et al., 1991); in fecal extracts, data were expressed as fecal testosterone on a per gram dry weight basis.

2.6. Biological validation

Sampling collection regimen: all urine and fecal samples were individually obtained from the total excreted by each animal over a 24 h period. A hCG treatment was administered to five males by injecting (s.c.) 2.5 IU/g body weight hCG (ELEA) in 0.9% sterile saline. Samples were collected 4 days before and 8 days after hCG treatment.

Concentrations of immunoreactive urinary or fecal androgen metabolites were compared between adult males and females (8–48 months; $n = 10$ in each group); individual samples were collected only for 1 day (Brown et al., 1996; Billity et al., 1998; Monfort, 2003).

2.7. Statistical analysis

Values are expressed as mean \pm standard error of mean (S.E.M.). The following tests were applied: analysis of variance (hCG challenge), in which animals of two-way ANOVA

represent blocks and factor treatment (days) at 12 times (4 days before and 8 days after hCG injection) and DGC (Di Rienzo et al., 2002) for testing the differences between daily means. Hormonal differences among sexes: urinary androgen metabolites and fecal testosterone were analyzed by Student's *t*-test (total amount of androgen/24 h; previously data were transformed to logarithms) or Wilcoxon test (androgen indexed for creatinine). Pearson's correlation coefficient was used to evaluate the relationship between urinary and fecal metabolites measurements. For all analyses, significance was assessed at least for the 0.05 level. All tests were conducted with Infostat (2000. Infostat version 1.1, Grupo Infostat, FCA-UNC, Argentina).

3. Results

3.1. Radioinfusion of testosterone

Total collected (7 days) urine volume and fecal weight were 44.0 ± 11.8 mL and 34.0 ± 2.7 g ($n = 4$ males), respectively. Radioactivity recovered from the administered ^{14}C -testosterone was $78.7 \pm 13.9\%$ over the experimental period. From that, $84.7 \pm 4.2\%$ of radiolabelled metabolites were excreted into urine and $15.2 \pm 3.9\%$ into feces. Urinary and fecal processing efficiency control resulted in $97.7 \pm 2.0\%$ ($n = 6$) and $92.5 \pm 10.67\%$ ($n = 6$) of recovered metabolized ^{14}C -testosterone, while the recovery of exogenous steroid was higher than 95.0 % in urine or in feces.

As depicted in Fig. 1, urinary excretion of ^{14}C -testosterone metabolites peaked at 8.2 ± 2.5 h ($n = 4$), while fecal excretion peak occurred later in all animals. A total of $82.7 \pm 4.2\%$ of urinary and $45.7 \pm 13.6\%$ of fecal radioactivity was excreted over the 24 h period after isotope administration. When the proportion of water-soluble (conjugated) or ether-soluble (unconjugated) forms of peak samples were evaluated, 100% of urinary and 98% of fecal metabolites were excreted in the aqueous phase. High-pressure liquid chromatography analyses indicated that there were several urinary metabolites; because profiles from samples containing radioactive peaks were quite similar, one of the profiles was chosen to evaluate the results (Fig. 2, panel A). None of the radioactive urinary metabolites derived from ^{14}C -testosterone co-eluted with the reference testosterone tracer, which is an unconjugated steroid. For feces (Fig. 2, panel D), three metabolites were separated; two were found in the aqueous phase and the other co-eluted with the ^{14}C -testosterone reference tracer. The fractions from both profiles depicted in Fig. 2, panels A and D, were analyzed by RIA. All three water-soluble peaks from urine (eluting at fraction 15–17, 18–20 and 32–34) crossreacted with the antibody. In feces, however, one peak (in the same elution area that the tracer) reached 11.8 ng/mL, whereas the others crossreacted minimally (under 0.2 ng/mL).

The dose–response curves for parallelism test (Fig. 2, panels B and E) and accuracy assessment (Fig. 2, panels C and F) clearly indicate that immunoreactive androgen metabolites were measurable in urine or feces, respectively. The standard curve of serially diluted calibrators ($y_1 = 72.80 - 0.04x$) was found to parallel the urine curve of serially diluted samples ($y_2 = 87.30 - 0.03x$). The same variables analyzed in feces were also parallel and reached the following values: $y_1 = 70.42 - 0.03x$ and $y_2 = 62.49 - 0.02x$,

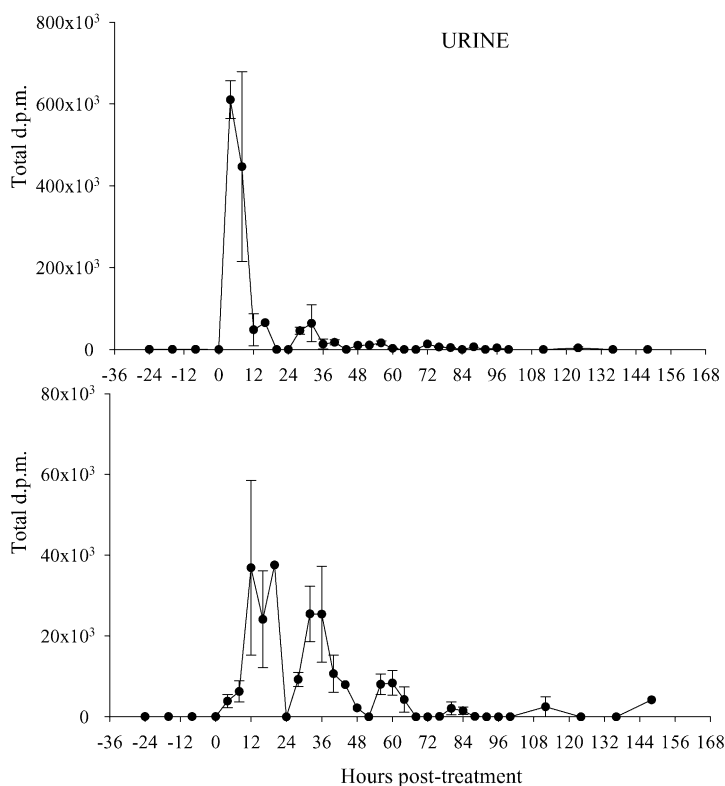


Fig. 1. Profiles of the excretory time-course of ^{14}C -testosterone in urine and feces after isotope administration (i.m.) at time 0 in chinchilla males ($n = 4$).

respectively. Furthermore, quantitative recovery of testosterone resulted in: $y = -11.42 + 0.80x$, $r^2 = 0.97$ for urine, and $y = 15.31 - 1.06x$, $r^2 = 0.99$ for feces.

3.2. Biological validation

Concentrations of immunoreactive urinary androgen metabolites increased three-fold during the first 3 days after hCG treatment; then, hormone concentrations were greater ($p < 0.05$, $n = 5$) for the following 3 days; finally, it decreased and reached initial concentrations by days 7–8 (Fig. 3, panel A, solid line). The same determinations indexed for creatinine are depicted (Pearson coefficient = 0.32, $p = 0.06$). The Fig. 3, panel B, depicts the results obtained in feces. When data are analyzed, a significant influence of each animal (“block factor”) becomes evident ($p < 0.05$). In fact, only two animals regularly defecated during the experimental period. Fecal testosterone from these two chinchillas increased from day 1 to day 6 after hCG injection ($p < 0.05$ as compared with days –4 to 0 and compared with days 7 and 8). A significant correlation was detected with respect to urinary androgen metabolites/day or indexed for creatinine ($r = 0.62$, $p = 0.002$).

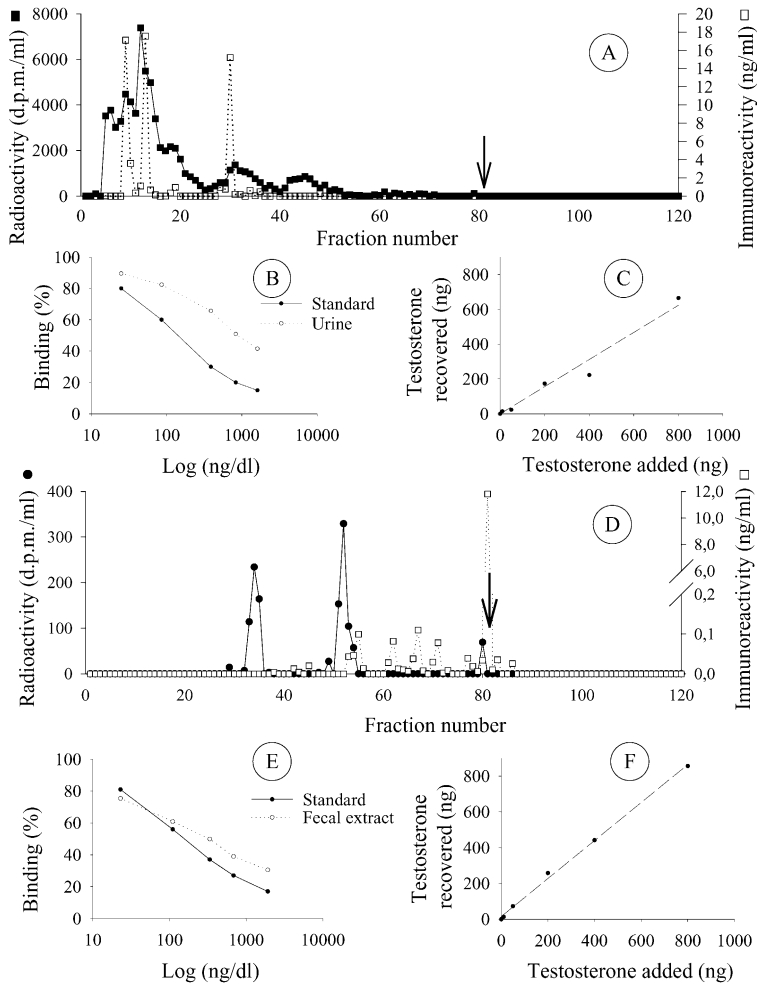


Fig. 2. High-pressure liquid chromatography separation of male chinchilla urinary (panel A, ■) and fecal (panel D, ●) androgen metabolites from peak samples after radiolabel infusion. One ml/min fractions were collected and testosterone immunoassay indicated immunoreactivity for different metabolites (urine, panel A; feces, panel D; □). Retention time of radioactive, and immunoreactive peaks were compared to ^{14}C -testosterone reference tracer (arrow). Serially diluted pooled male chinchilla urine (panel B) or feces (panel E) samples binding to antibody in parallel with testosterone serially-diluted standards. Quantitative recovery of exogenous testosterone from male chinchilla urine (panel C) or feces (panel F). Each point represents three replicates evaluated in the same assay.

Finally, the immunoreactive urinary androgen metabolite concentrations were 28.70 ± 6.96 ng/day ($n = 10$) and 9.08 ± 1.45 ng/day ($n = 10$) ($p < 0.05$) in untreated males and females, respectively; results indexed for creatinine indicated the same differences ($p < 0.05$). In feces, 64.33 ± 8.37 ng/day in males ($n = 10$) and 68.77 ± 8.38 ng/day in females was excreted ($n = 10$).

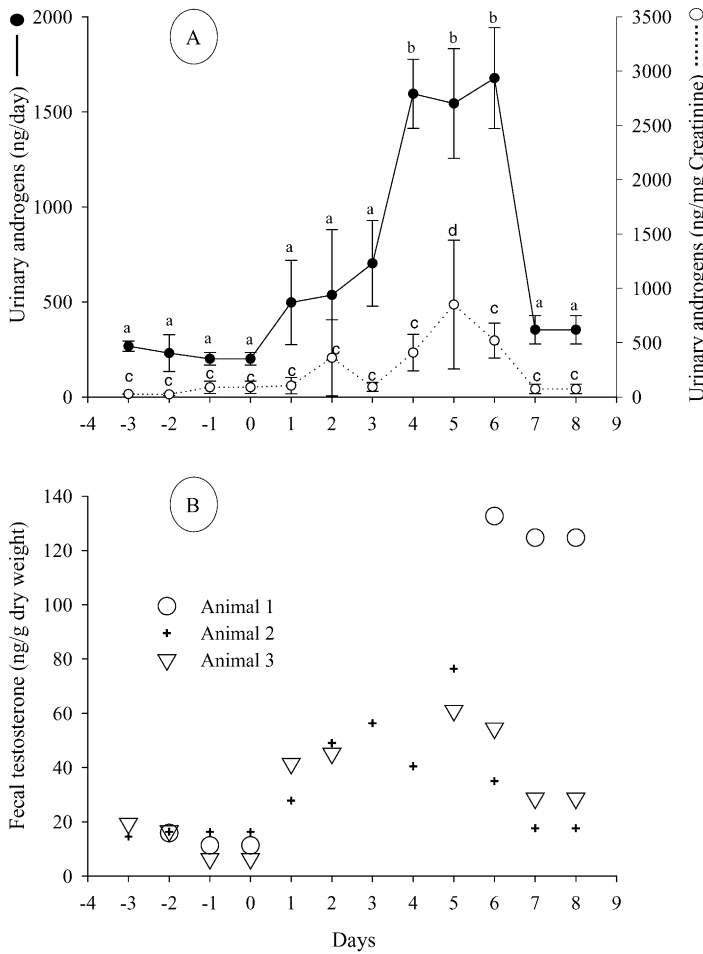


Fig. 3. Effects of hCG treatment (s.c., 2.5 IU/g body weight at day 0) on the secretion of androgen metabolites of male chinchilla. Panel A: Urine; data are expressed as ng/day (solid line) and as ng/mg creatinine (dotted line); a as compared with b, and c as compared with d: $p < 0.05$ ($n = 5$). Panel B: feces ($n = 3$).

4. Discussion

The present study provides basic information on the excretion of testosterone in *C. lanigera*. Results clearly show that, after ¹⁴C-testosterone treatment, the hormone is rapidly metabolized and predominantly excreted into urine. After separation of several metabolites in urine and feces, the measurements of such metabolites were validated. A commercially available testosterone radioimmunoassay was effective in detecting hormone changes in this species; on the basis of this evidence the procedures were validated for future studies in chinchillas.

Several reports have demonstrated that, in mammals, urinary and/or fecal steroid hormone metabolites can be used to monitor gonadal functions (Palme et al., 1996; Heistermann et al., 1996; Schwarzenberger et al., 2000; Muir et al., 2001; Ginther et al., 2002). Previous studies on steroidal hormone metabolism performed in other mammals indicated that most of the urinary and fecal metabolites are excreted within 24 h (Monfort, 2003). Similarly, results in the present study indicate that 24 h after radioisotope administration almost all urinary metabolites and at least 30% of fecal metabolites were excreted. Previously, the excretion pattern of corticosteroids in this species was reported with the same methodology (Ponzio et al., 2004). With respect to testosterone, the time course of metabolite excretion was similar to that reported by Billity et al. (1998) in rodents such as *Mus musculus* and *Peromyscus maniculatus*.

When results are assessed for the main route of steroidal metabolites excretion, there are inconsistencies. Mice, rats and *Peromyscus* spp. excrete up to 60% of testosterone metabolites in feces, while guinea pigs and rabbits excrete more than 80% in urine (Taylor, 1971; Billiti et al., 1998). In chinchilla, we established that the urinary route was predominant in males. These results suggest that there exists a preferential route of steroidal metabolite excretion and that this varies depending on the rodentia suborder; therefore, in Hystrichomorpha (chinchilla and guinea pigs) the urinary route is preferential while in Sciuomorpha (rats and mice) and in Myormorpha (*Peromyscus* spp.) the feces is the primary excretory route.

The predominance of conjugate urinary steroids and unconjugated fecal steroids was found in some farm mammals by Palme et al., (1996) and reviewed in some wild animals by Monfort (2003). Steroidal metabolites are de-conjugated by intestinal flora and excreted with feces. However, in the present study testosterone metabolites were predominantly conjugated not only in urine but also in feces. Similar results were obtained in rodents (Billiti et al., 1998), lagomorphs such as hares (Teskey-Gerstl et al., 2000) and in felids (Brown et al., 1994, 1995, 1996).

In urine, HPLC analyses confirm that most of the metabolites were conjugated, and also revealed that none were associated with the reference testosterone tracer. Concerning the immunoreactivity of these urinary metabolites, several ^{14}C -testosterone peaks were detected in the HPLC fractions. These metabolites could be useful for measuring testicular activity despite the cross-reactivity because, according to the immunoassay used in the present study, these metabolites are structurally related to testosterone. From a practical viewpoint, it is unnecessary to determine the specific molecular structure of hormonal metabolites; however, it is important to prove that fluctuations in such metabolites provide physiologically relevant information (Monfort, 2003).

It is remarkable that, after separation by HPLC, there was a difference between excreted metabolites in urine or feces; according to immunograms, only one (presumably unconjugated) of three fecal metabolites of ^{14}C -testosterone was detectable with the immunoassay. This radioactive peak co-eluted with testosterone tracer. From a comparative perspective, therefore, HPLC immunograms (urine as compared with feces) clearly illustrate that, using the RIA used in the present study, four primary metabolites were detected, but only one reliably indicates the presence of testosterone.

Concerning the performance of the total testosterone RIA used in the present study, the analytical assessment demonstrated that the immunoassay was capable of detecting immunoreactive changes in urinary androgen and fecal testosterone; biochemical validation

(precision, specificity and accuracy) were quite similar, suggesting a greater recovery for fecal extracts (analysis of regression = $15.31 + 1.06 \times \text{hormone mass}$, $r^2 = 0.99$).

Our final objective was to evaluate changes in urinary androgen and fecal testosterone metabolite excretion and their relationship after treatment with a hormone that induced testosterone release (hCG). The experiment demonstrated that males excreted a greater amount of urinary androgen as well as fecal testosterone metabolites. Similarly, in mice, the basal amount of fecal testosterone increased after hCG treatment (Billiti et al., 1998). Results from the present study also indicate (as a result of treatment with hCG) that urinary determinations are correlated with those for fecal testosterone; indeed, from the comparison of Figs. 1 and 3, there is a clear indication that the time course and concentrations of all immunoreactive metabolites resemble those ^{14}C -forms excreted after radioisotope administration in other groups of males.

The feces assessments indicated an “animal effect”; this effect evoked a delayed excretion of testosterone metabolites. Constipation has previously been detected in other farm animals (Palme et al., 1996). Thus, the time delay in the present study cannot likely be attributed to different metabolism and excretion of the steroids.

To support the biological relevance of the validated assay, the significant differences in urinary androgen metabolite excretion between males and females constitute additional evidence. Nevertheless, these differences were not evident in fecal samples. The reliability of 24 h determination of fecal testosterone, therefore, requires further investigation.

In conclusion, after ^{14}C -testosterone treatment in domesticated *C. lanigera*, the hormone is metabolized in 24 h and excreted predominantly in urine. The immunoreactivity of several ^{14}C -androgen metabolites in urine and in feces were assayed; a single metabolite from fecal extracts would be considered as testosterone. Based on data from the present study, all these immunoactive metabolites should be useful to non-invasively assess testicular activity by detecting hormone changes in excretions with the validated radioimmunoassay for testosterone. Finally, the present study constitutes an advance in knowledge on chinchilla male reproductive biology and in application of assisted reproduction techniques on this endangered species (CITES) and their farmed counterparts.

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