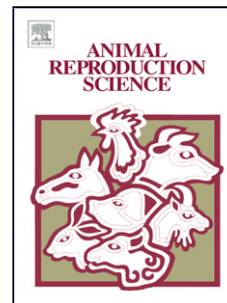


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ONE YEAR DAILY CHANGES IN FECAL SEXUAL STEROIDS OF TWO CAPTIVE
FEMALE CHEETAHS (*ACINONYX JUBATUS*) IN ITALY.

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HIGHLIGHTS

- Non-invasive monitoring of sexual steroid in captive sibling cheetahs
- Daily fecal collection throughout one year of females housed in the same conditions
- RIA on wet feces
- Temporal pattern of ovarian dynamics

ABSTRACT

The present study evaluated changes of fecal sexual steroids in two female cheetahs (Geijsha and Duchessa) in Northern Italy throughout one year. Wet feces were collected daily from two sibling animals of the same age, housed with conspecific males and managed in the same conditions, and estrogens and progestogens concentrations were analyzed by radioimmunoassay (RIA).

Evidence of ovarian activity based on regular fluctuation in estrogen excretion was demonstrated in both females. None of the animals was continuously cycling, as follicular activity was interrupted by anestrus periods, during the spring and early winter. No significant increases of progestogens were recorded after the estrogen peaks, indicating that induced or spontaneous ovulations did not occur during the observation period. The wavelet decomposition evidenced the temporal pattern of ovarian activity in the two females, underlying throughout the year a more pronounced rhythmical ovarian estrogenic activity in Geijsha than in Duchessa. However, this statistical approach had a smoothing effect in depicting the hormonal patterns and the number of follicular phases might be lower than that revealed by the iterative method.

In this study, RIA on wet feces performed very well to determine sexual steroid concentrations, and an ovarian activity interrupted by anestrus periods along the year in captive cheetahs co-housed in a small group was demonstrated.

More information on estrous behavior of captive cheetahs were obtained in this study, but the effects of husbandry and management conditions on natural reproductive physiology of this species remain to elucidate.

Key words: *Acinonyx jubatus*; fecal steroids; RIA; ovarian activity

INTRODUCTION

The International Union for Conservation of Nature (IUCN, www.iucnredlist.org) classified cheetah (*Acinonyx jubatus*) as vulnerable wild species due to population decrease for habitat destruction, poaching and conflicts with ranching (Myers, 1975). In captivity, cheetah's reproductive performances are poor, mostly because of inappropriate husbandry, management and inability to identify the female estrous timing (Brown et al., 1996; Pelican et al., 2006).

Cheetahs are non-seasonal polyestrous felids with short estrous cycles (range from 7 to 21 days). They start breeding at 2–3 years of age (Bertschinger et al., 2008; Crosier et al., 2011) and cycle approximately every 12 days, with estrous phases of 2-6 days and anestrus of 2-5 months. Mating-induced ovulations are typical of this species, spontaneous ovulations were also documented although at very low incidence (Asa et al., 1992; Brown et al., 1996). The duration of non-pregnant luteal phase is about 53 days and the gestation lasts 94 days, with 3-4 cubs (Brown et al., 1996; Brown, 2011). In captive animals, variability in ovarian quiescence for a so called “stress-associated ovarian suppression” (Brown et al., 1996; Jurke et al., 1997) and a variable male influence on the female cyclicity and estrous behavior were observed (Brown et al., 1996; Kinoshita et al., 2011).

As the captive propagation is currently considered one of the major conservation tools, a well-known understanding of cheetah's reproductive behavior and physiology is needed for achieving breeding success or for the application of assisted reproductive programs when the natural breeding fails (Wielebnowski and Brown, 1998).

The measurement of plasma gonadal steroid levels is the most accurate method to evaluate ovarian hormonal secretion, but repeated blood sampling is impractical in wild and non-collaborative animals. A non-invasive monitoring of endocrine status can be performed by measurement of steroid metabolites in urine and/or feces in different species (Biancani et al., 2009; Lasley and Kirkpatrick, 1991). In felids, because sexual steroids are excreted mainly in feces, and urine is voided by spraying and hard to collect in the field, the extraction from fecal samples is the elective method (Graham et al., 1995; Shille et al., 1990). The monitoring of estrous cycle and diagnosis of early pregnancy through the determination of fecal sexual steroids have been documented in captive cheetahs, but the different housing and managing conditions of animals, the different frequency of fecal sampling and of duration of whole experiments have produced variable results (Brown et al., 1996; Kinoshita et al., 2011; Koester et al., 2017ab).

Therefore, the aim of this study was to evaluate changes of sexual steroids in two captive cheetahs housed in a small group with conspecific males in the North of Italy. Wet feces of each animal were collected daily for a whole year and estrogens and progestogens concentrations were analysed by radioimmunoassay (RIA).

MATERIALS AND METHODS

Animals and fecal samples collection

Two siblings female cheetahs (Geijsha and Duchessa) housed at *Le Cornelle Animal Park* (Valbrembo, BG, Italy; 45°42' N 9°35' E) were included in this study. The experiment started when animals were 2 years old and was concluded at the end of their third year of age. Housing conditions were unchanged throughout the study. During the day, the females were housed with others 3 conspecific males, whereas

in the night they were separated in single and different cages, with the possibility of visual and olfactory contacts. All the animals were exposed to natural photoperiod.

Fecal samples were collected daily by animal keepers from each female between 7 am and 9 am for one year, collecting a total of 312 for Geijsha and 308 samples for Duchessa. Fecal samples were placed in plastic boxes and stored at -20°C until processed for hormonal determinations.

Fecal steroid extraction

The steroid extraction was performed from wet fecal samples following the double extraction technique described by Brown and co-workers (1994) for dry samples, with some modifications. Wet fecal samples (0.2 g) were extracted with 5 ml 90% ethanol. The mixture was carefully vortexed for 10 min at room temperature, boiled for 20 min and centrifuged at 1,300 g for 10 min. The ethanolic supernatants were recovered and transferred in a fresh glass tubes. The pellets were extracted again with 5 ml of 90% ethanol by vortexing 5 min at room temperature and centrifuged (1,300 g for 10 min). Both ethanolic supernatants were combined (total volume: 10 ml) and dried completely under N_2 flow at 37°C . The dry extracts were dissolved in 1 ml of absolute ethanol by vortexing for 5 min at room temperature. Aliquots (50 μl) of the ethanolic extracts were dried under N_2 flow and dissolved in 250 μl of absolute methanol. Ten μl /well and 20 μl /well were added in duplicate for the estrogen and progesterone radioimmunoassay (RIA), respectively (Fig. 1).

Estrogen and progesterone Radioimmunoassays (RIA)

Sexual steroid concentrations were analyzed by solid-phase microtiter RIAs as previously described in bottlenose dolphins (Biancani et al., 2009). Briefly, 96-well microtiter plates (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA) were coated with a goat anti-rabbit γ -globulin serum diluted 1:1000 in 0.15 mM sodium acetate buffer pH 9 and incubated overnight at 4 C. The plates were carefully

washed with RIA buffer (61 mM di-sodium hydrogen orthophosphate, 40 mM sodium di-hydrogen orthophosphate, 154 mM sodium chloride, 0.1% BSA, pH 7.4), followed by the addition of 0.2 ml of either rabbit anti-P4-carboxymethyloxime-BSA serum (working dilution 1:8000) or rabbit anti-E2-6-carboxymethyloxime serum (working dilution 1:15,000) dissolved in RIA buffer. After an overnight incubation at 4°C, the antiserum solutions were decanted, plates were washed with RIA buffer, and standards, quality controls, and unknown extracts (estrogens: 20 µl/well; progestogens: 10 µl/well) were added in duplicate. For estrogen analysis, the standard curve was made by serially diluting (1.5 to 200 pg/well) an estrogen preparation (Sigma) in RIA buffer. The tracer [2,4,6,7-³H]estradiol (Perkin-Elmer Life and Analytical Sciences; specific activity, 72 Ci/mmol) was used at 30 pg well⁻¹ 10 µl⁻¹. For progestogen analysis, the standard curve was made by serially diluting (2.5 to 320 pg/well) a progesterone preparation (Sigma, Chemical Co., St. Louis, MO, USA) in RIA buffer. [1,2,6,7-³H]Progesterone (Perkin-Elmer Life and Analytical Sciences; specific activity, 97 Ci/mmol) was used as the tracer at concentration of 30 pg well⁻¹ 10 µL⁻¹. The total volume of the reaction mixture was adjusted to 0.2 ml with RIA buffer and was incubated overnight at 4 C. Finally, the reaction mixture was decanted, and the plate was carefully washed. Bound radioactivity was β-counted (Top-Count; Perkin-Elmer Life and Analytical Sciences).

Cross-reactions for the estrogens antiserum were 17β-estradiol, 100%; estrone, 2.5%; estriol, 1.2%; and <0.007% for the other tested steroids. The antiserum did not cross-react with conjugated estrogens.

Cross-reactions for the progestogen antibody were progesterone, 100%; 11α-hydroxyprogesterone, 77%; 11β-hydroxyprogesterone, 65%; 17α-hydroxyprogesterone, 2.9%; 20α-hydroxyprogesterone, 0.01%; and 20β-hydroxyprogesterone, <0.001%. The antiserum did not cross-react with conjugated progestogens.

The sensitivity of the assays was 1.5 pg/well and 2.5 pg/well for estrogen and progestogen RIAs respectively.

Parallelism and repeatability tests were performed for both estrogen and progesterone RIAs. Parallelism was assessed by calculating the regression curve between the observed hormone concentrations and the reciprocal of the dilution factors in serially diluted (1:2 to 1:16) fecal extracts. The intercept of the parallelism curve not significantly different from 0 indicates a good degree of parallelism. The repeatability of the estrogen RIA was assessed in samples containing high and low estrogen concentrations (approximately 50 and 10 ng/g). The intra-assay coefficients of variation were 3.44% and 5.64% for the high and low estrogen samples, respectively, whereas the inter-assay coefficient of variation was 6.65%. The repeatability of the progesterone RIA was evaluated in samples containing high and low progesterone concentrations (approximately 80 and 40 ng/g). The intra-assay coefficients of variation were 9.59% and 1.89%, for the high and low progesterone samples, respectively, whereas the inter-assay coefficient of variation was 8.89%.

Statistical analysis

The statistical analysis was performed following the method described by Brown et al. (1996). Briefly, basal estrogen concentrations were determined by an iterative process in which, after the calculation of mean \pm standard deviation (SD), values that exceeded the mean of +1.5 SD were excluded. Data over the mean value of 1.5 SD were indicative of estrous period and, among them, the values that exceeded preceding values by 50% were considered peak of estrogen concentrations. The duration of periods of follicular activity was calculated as the number of days between two different estrogen peaks. Peak intervals between 5-30 days were evaluated and estrogen peaks intervals >30 days were considered as anestrus periods. Basal progesterone concentrations were calculated from the mean values of 7 days preceding the peak of estrogens. Postovulatory increases in progesterone excretion were considered as significant if values exceeded the mean +2 SD of the preceding values and remaining high for at least 1 week.

Data of hormonal concentrations are presented as means \pm SD and follicular phases were partitioned by percentiles. Differences between animals were analyzed by D'Agostino & Pearson normality test and Unpaired t test. A level of significance was set at $p < 0.05$.

To evaluate changes of hormonal secretion, temporal patterns for fecal estrogens and progestogens in the two cheetah females were analyzed through wavelet analysis decomposition using as mother wavelet the Morlet wavelet with sample interval=1 (Cazelles et al., 2008). Wavelet decomposition is a time series analysis method derived from Fourier harmonic analysis; Fourier approach is based on the decomposition of a complex signal into harmonic components (Cazelles et al., 2008). This analytical method assumes the stationarity of a given signal over time, while wavelet analysis overcomes this pitfall performing a time-scale signal decomposition. In this way, the signal can be examined in several period components varying with time (Cazelles et al., 2008). In the present work, decomposition results were depicted on a day vs. period graph, expressed as \log_2 values. For each decomposition process, the $p < 0.05$ power levels were extracted for each hormone, and the significant power peaks were calculated in terms of periods in days \pm SD. The wavelet analysis was performed with the software Past 3.16 for Windows platform (freely downloadable at website <http://folk.uio.no/ohammer/past/>).

RESULTS

The intercepts of the parallelism curves for the fecal estrogen ($[Hobs] = 23.23 [1/fd] + 1.208; R^2 = 0.99$) and fecal progestogen ($[Hobs] = 312.21 [1/fd] - 1.173; R^2 = 0.99$) RIAs were not significantly different from 0.

Evidence of ovarian activity based on regular fluctuation in estrogen excretion was demonstrated (Fig. 2 and 4). During one-year evaluation, 13 phases of follicular activity in Geijsha and 10 in Duchessa were observed, with a duration of 10.3 ± 3 days (range 6-15) and 11.4 ± 6.9 days (range 5-23), respectively.

When partitioned by duration, the percentage of these periods <7, 8-13, 14-19 and >20 days in length were 30%, 48%, 13% and 9%, respectively, without significant differences between the two females or within the same animal ($p=0.45$). Baseline estrogen concentration was 22 ± 6 ng/g for Geijsha and 19 ± 5 ng/g for Duchesssa; peak estrogen concentration (17 for Geijsha and 13 for Duchesssa) ranged from 31 to 74 ng/g and from 30 to 165 ng/g, respectively. None of the animals was continuously cycling, as follicular activity was interrupted by anestrus periods. Geijsha showed 3 anestrus phases of 46, 48, and 82 days, whereas Duchesssa showed 2 anestrus phases of 73 and 115 days (Fig. 2 and 4).

The values of basal progestogens were 113 ± 42 ng/g in Geijsha and 101 ± 23 ng/g in Duchesssa. As showed in Fig. 3 and 5, no significant increases in fecal progestogen concentration were observed after estrogen peaks, thus demonstrating that any ovulation occurred. In addition, no estrus behavior or sexual interest of males were noticed.

The Morlet wavelet decomposition evidenced changes of fecal hormone concentrations (Fig. 6). In Geijsha, a low-frequency temporal pattern was observed during the whole observation period for both fecal estrogens and progestogens. These temporal patterns appear as red strips in the lower margin of the graphs (Fig. 6a c). Estrogen concentrations showed three significant ($p<0.05$) zenith, every 31.5, 62.0, and 101.7 days (Fig. 6a). Geijsha fecal progestogen wavelet decomposition was characterized by two significant ($p<0.05$) zenith, at 36.9 and 107.3 days (Fig. 6c). In Duchesssa, only two high-power estrogen periods were observed: a medium-frequency period at 37.2 days, and a low-frequency period, at 81.7 days (Fig. 6b). The appearance of a significant power period was limited to the time interval of 0-160 days. In fecal progestogen concentrations, a significant low-frequency pattern ($p<0.05$) lasting almost the whole observation period (mean 105.2 days) and a weak, significant pattern at 39.5 days were observed (Fig. 6d). Moreover, wavelet decomposition explores variations in hormone patterns, allowing the analysis in periods not dominated by constant oscillations. Then, in the upper side of the graph (Fig. 6) the periodic patterns in high frequency, and in the lower side the low-frequency patterns are

represented. In this way, several patterns with different frequency are visible (orange/red areas), and periods with the higher ovarian activity are evidenced.

DISCUSSION

The present study evaluated changes of concentrations of fecal sexual steroids in two female cheetahs housed in Northern Italy. The determination of fecal steroids to monitor the ovarian activity has already been performed in cheetahs, but this is the first time that fecal samples were collected daily throughout one year from two sibling animals of the same age, housed and managed in the same conditions.

The RIA already used to measure estrogens and progestogens in fecal extracts from bottlenose dolphins (Biancani et al. 2009) performed very well in measuring fecal sex steroid concentrations in female cheetahs. The parallelism was good for both the fecal estrogen and progestogen assays, and both assays could detect the hormone across a wide range of dilutions. Both assays showed a good degree of precision.

Fecal sex steroid concentrations differ between studies, and this may depend upon the characteristics of the extraction and immunoassay methods employed. In this study, steroid extraction was performed by boiling wet fecal samples in ethanol with 10% water (Brown et al., 1994), which allowed the efficient extraction of both conjugated and un-conjugated steroid metabolites. Then, the extracts were analyzed by specific RIAs.

The fecal estrogen concentrations observed here are comparable with those reported by others (Brown et al., 1994, 1996), who used a RIA with an antibody specific for 17 β -estradiol but showing negligible cross-reactivity for conjugated estrogen metabolites. Likewise, the antiserum used in this study was very

specific for the native 17β -estradiol and without cross-reactivity for conjugated estrogens; therefore, fecal estrogens are likely represented mostly by native 17β -estradiol. Indeed, fecal estrogen concentrations were dramatically higher when fecal extract had been analyzed using an immunoassay with an antibody cross-reacting with conjugated estrogens (Kinoshita et al., 2011).

Conversely, fecal progesterone concentrations reported in this study are far lower than reported by others (Adachi et al., 2011; Brown et al., 1994, 1996; Kinoshita et al., 2011). In cheetahs, circulating progesterone is almost completely metabolized and it is excreted in feces mostly as reduced 5α -pregnane metabolites; while native progesterone is barely present (Adachi et al., 2011; Brown et al., 1994). The antiserum employed in this study showed poor, if any, cross-reactivity with 5α -pregnane metabolites and was not able to detect conjugated progestogens. Therefore, it is conceivable that this assay could detect the little native progesterone and, perhaps, 11α , 11β and 17α metabolites possibly present in cheetah's fecal samples.

Several variables could affect the concentration of fecal hormones (e.g.: water content, diet, time of collection after defecation, environmental exposure, storage time and temperature, sample volume, consistency and homogeneity; Wielebnowski and Watters, 2007). It is widely accepted that the use of dry feces reduces the intrinsic variability of samples, if a well-mixing is performed before extraction. However, under certain circumstances the use of wet feces can be preferable because it reduces the working time and simplifies the whole procedure; indeed, reasonable data can be obtained with well-mixed wet feces, when animal diets and health remain constant (Brown, 2011). Present results evidenced that the fecal samples collected directly from healthy subjects showed a low variability in water content (range 64.8 - 75.1%; $70.4 \pm 4.1\%$; mean \pm SD). The variability (CV = 5.7%) between samples was in the range usually observed in immunoassays.

In the present study, fecal estrogen and progesterone concentrations revealed that in cheetahs the follicular activity lasts on average 10-11 days, as estimated by others by fecal and plasma steroid concentrations (Bertschinger et al., 1984, Brown et al., 1994,1996, Czekala et al., 1994, Graham et al., 1995, Kinoshita et al., 2011), behavioral observation (Wielebnowski and Brown, 1998, Wielebnowski et al., 2002) or vaginal cytology (Asa et al., 1992). However, the two cheetahs showed individual follicular phase variability, with a different range in days (6-15 for Geijsha and 5-23 for Duchessa) as reported elsewhere (Brown et al., 1994, 1996; Czekala et al., 1994; Kinoshita et al., 2011).

Neither females showed behavioral signs of estrus, nor co-housed males showed any interest in females. In addition, no significant increases of progesterone were recorded after the estrogen peak, indicating that induced or spontaneous ovulations did not occur during the observation period.

The average duration of anestrus was consistent with previous results (Brown et al., 1996), with some differences among animals. Geijsha showed shorter anestrus and more follicular phases compared to Duchessa. Brown (2006) suggested that ovarian activity might be inhibited when female cheetahs are kept in pairs. Low fecal estrogen concentrations and poor cyclic activity were observed in subordinate females, but several external factors, as pair composition, behavioral compatibility and captivity stress must be also considered as concurrent causes (Wielebnowski et al., 2002). However, recent findings reported that the presence of small familiar groups of female cheetahs or the presence of adult conspecifics (males and/or females) in nearby enclosures did not exert any valuable influence on ovarian and adrenal hormonal concentrations (Koester et al., 2017).

It is noteworthy that the two females of the present study showed similar anestrus period during the spring and early winter (April-June and November-December).

The wavelet decomposition evidenced the temporal pattern of ovarian activity in the two females. An underlying rhythmical ovarian estrogenic activity, which was more pronounced in Geijsha than in

Duchessa was observed throughout the year. Conversely, the temporal trend of progesterone were similar in the two cheetahs with a low frequency pattern throughout the whole observation period.

This statistical approach had a smoothing effect in depicting the hormonal patterns, and it may lead to the identification of a lower number of follicular phases in comparison with the iterative method. Indeed, the wavelet decomposition evidenced a lower ovarian activity in the time intervals in which some estrogen peaks were observed by the iterative method (Geijsha in October-December, Fig. 6a; Duchessa in November-January, Fig. 6b). By the iterative method, estrogen peaks are identified by taking into account the preceding value, while the wavelet decomposition method analyzes the time as a *continuum*. Therefore, this statistical method might be considered as complementary to the iterative method, since it generates other detailed information.

In the cheetahs herein examined, although waves of estrogen excretion and occasional periods of anestrus were observed, no reproductive behavior occurred. This finding supports the fact that a non-natural management of captive animals, as keeping together males and females continuously as in the present study, is probably the main cause of breeding failure in this species (Beekman et al., 1997; Brown et al., 1996). Female cheetahs are known to be solitary and meet males only during the reproductive period. Therefore, the cohabitation in *ex-situ* conditions (zoo or animal parks) or the absence of stimuli could negatively influence their reproductive activity (Wielebnowski et al. 2002). Conversely, other authors stated that managing cheetahs in small cohorts and in limited space does not negatively affect their reproductive performances (Koester et al., 2017). These contradictory results derived from observation made in different conditions, as to standardize captivity management is hard.

CONCLUSIONS

In conclusion, sexual steroids extracted from wet feces demonstrated an ovarian activity interrupted by anestrus periods along the year in captive cheetahs co-housed in a small group. Effect of husbandry

and management conditions, i.e. housing in groups or as singletons, with or without environmental stimuli, deserves further investigations to update the information on cheetah's reproductive physiology and to increase their reproduction behavior in captivity.

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Author contributions.

VV collected the data and drafted the paper. MGM and GCL contributed to analyse the data and drafted the paper, GG and LDD performed the RIA analysis and collaborated in drafting the paper. All authors have approved the final version.

Conflict of interest.

Conflicts of interest: none

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FIGURE CAPTIONS

Figure 1. Description of the extraction procedure of sexual steroids from wet fecal sample.

Figure 2. Representative individual longitudinal profiles of fecal estrogen concentrations in the female cheetah Geijsha.

Asterisks denote peaks in estrogen excretion significantly above the baseline; line bars denote periods of anestrus.

Figure 3. Representative individual longitudinal profiles of fecal progestogens concentrations in female cheetah Geijsha.

No evidences of ovulation were observed.

Figure 4. Representative individual longitudinal profiles of fecal estrogen concentrations in the female cheetah Duchessa.

Asterisks denote peaks in estrogen excretion significantly above the baseline; line bars denote periods of anestrus.

Figure 5. Representative individual longitudinal profiles of fecal progestogens concentrations in female cheetah Duchessa.

No evidences of ovulation were observed.

Figure 6. Patterns for fecal estrogens (a,b) and progestogens (c,d) wavelet decomposition and mean power peaks in Geijsha (a,c) and Duchessa (b,d). In wavelet decomposition, the cone of interest and the $p < 0.05$ areas (yellow-red) are reported.

