Public Exposure and Number of Conspecifics have no Influence on Ovarian and Adrenal Activity in the Cheetah (*Acinonyx jubatus*)

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Abstract

Cheetahs in managed zoological collections do not reproduce efficiently, a problem that may be related to environmental/management stressors. In this study, we examined 17 adult female cheetahs to determine the influence of two environmental factors, (1) being housed on- or off-exhibit and (2) number of adult conspecifics (males and/or females) in nearby enclosures, on profiles and concentrations of ovarian and adrenal hormones. Secondly, we assessed a subset of group-housed siblings ($n = 5$ in groups of 2 or 3) for effects of long-term cohabitation. All of the females demonstrated waves of estrogen excretion (indicative of ovarian activity) as well as occasional periods of no estrogen production (anestrus). Glucocorticoid and estrogen concentrations were correlated within an individual ($r_s = 0.56$; $P < 0.05$), and overall there was a higher frequency of days with elevated glucocorticoid concentrations in association with elevated estrogen excretion. However, none of the management factors had an impact ($P > 0.05$) on estrogen or glucocorticoid metabolite excretory patterns. Although we recently reported that public exposure can negatively affect sperm production, ovarian steroidogenesis in females was unaffected. There also was no evidence of hyper-adrenal activity. Thus, different methods of *ex situ* management appear to have minimal influence on ovarian function or stress susceptibility of female cheetahs.

Additional keywords: felid, management, steroid hormone

1. Introduction

Numbers of free-ranging cheetahs (*Acinonyx jubatus*) have decreased by more than 30% since the 1970s, and declines have resulted in estimates of <10,000 individuals in the wild today (Durant et al., 2015). Contemporary causes are mostly attributable to continued habitat loss and conflict with local farmers (Marker et al., 2003, 2008). Therefore, *ex situ* collections of cheetahs living in controlled conditions serve as both security (insurance) and as a resource for conducting hypothesis-based studies impossible to perform with cryptic, free-living counterparts. Cheetahs in North America are managed within a zoo cooperative called the Species Survival Plan (SSP; Grisham et al., 2013) that, besides striving for a demographically and genetically stable population, encourages basic research to expand fundamental knowledge. The amount of information generated through SSP collaborations and then published in the peer-review literature has been substantial, providing significant insights into species' reproductive physiology (Brown et al., 2001; Brown and Wildt, 1997; Crosier et al., 2006, 2009; Howard et al., 1992, 1997; Roth et al., 1995; Terrell et al., 2011, 2012; Wildt et al., 1988, 1993), behavior (Wielebnowski and Brown, 1998; Wielebnowski, 1999; Wielebnowski et al., 2002), nutrition (Dierenfeld, 1993; Vester et al., 2010), genetics (O'Brien et al., 1983, 1985, 1987), and disease susceptibility (Munson et al., 1999, 2002, 2005; Terio et al., 2004, 2012).

Significant gaps remain, however, in thoroughly understanding what drives successful female cheetah reproduction, with studies sometimes generating more questions than answers. First, rather than exhibiting felid-typical estrus (e.g., protracted periods of lordosis and strong vocalizations), overt signs of sexual receptivity in the cheetah often are brief, subtle, or

completely absent (Wielebnowski and Brown, 1998). Although pregnancy can sometimes be established after only a single, brief mating episode, many observed copulations (even with a proven breeder male) can fail to produce offspring in some individuals. For example, of 54 mating events involving different females within the SSP population in 2014 and 2015, ~60% failed to result in cubs. There are other oddities, including that females normally produce rather ‘abrupt’ waves of estrogenic activity every 5 to 30 days, with most peaks being unaccompanied by sexual behavior (Brown et al., 1996; Crosier et al., 2016; Wielebnowski and Brown, 1998). Cheetahs also inexplicably produce periods of complete reproductive inactivity for as long as 2 to 5 months before randomly re-initiating estrogen cyclicity (Brown et al., 1996; Crosier et al., 2016). These intervals of ovarian shutdown, or anestrus, are not synchronous among females and are unrelated to seasonality (Brown et al., 1996).

There are multiple reports on the independent monitoring of ovarian (Adachi et al., 2011; Brown et al., 1996; Wielebnowski et al., 2002) or adrenal (Crosier et al., 2016; Koester et al., 2015; Terio et al., 2004; Wells et al., 2004) hormone metabolite patterns in the feces of captive-held cheetahs. However, there has been little emphasis on the simultaneous assessment of both metabolites in adult females, especially to determine if measured glucocorticoids are related to estrogen profiles or affect ovarian activity. There are many examples in other mammals species, including via assessments of fecal glucocorticoids, demonstrating that ‘stress’ can influence female reproductive state (Touma and Palme, 2005). For the cheetah, physiological stress has been suggested as one mechanism for limiting ovarian activity in animals managed in captivity; such females have larger adrenal cortices that produce more excreted glucocorticoids than wild counterparts (Terio et al., 2004). Others

have focused on specific environmental perturbations, finding that exposure to a high number of human care-givers (Koester et al., 2015) and the transfer from off- to on-exhibit facilities (Wells et al., 2004) can increase glucocorticoid excretion in female cheetahs. Certainly, it makes sense that a specialized carnivore like the cheetah may be more reproductively successful in a non-urban zoo environment where there are fewer external distractions that may be perceived as stressors. This is one reason why most adult cheetahs now are managed under 'breeding center' conditions, in more spacious enclosures with little public exposure. There is evidence that such a strategy has applied benefits as a retrospective analysis of data from North American zoos reveals that >90% of cheetah litters in the last decade have occurred in these off-exhibit centers compared to traditional urban zoos (Grisham et al., 2013; Marker, 2012).

Besides exposure to the public in a traditional zoo environment, another factor of potential importance could be presence of nearby conspecifics. Wild female cheetahs normally live a solitary life-style, except for occasional interactions with males for mating or during the ~18 mo interval of rearing young (Caro, 1994). One study of cheetahs in captivity observed that the dual housing of unfamiliar/unrelated females in a single enclosure caused almost immediate ovarian activity shutdown, which was reversed by separating the pair into adjacent enclosures (Wielebnowski et al., 2002). The one outlier in that study was an adult mother-daughter pair that displayed high frequencies of affiliative behaviors while sustaining active ovarian function throughout the study. A similar observation was made in another long-term pair of female cheetahs, both of which also sustained normal ovarian cyclic patterns (Kinoshita et al., 2011).

Our team recently discovered that male cheetahs housed off-exhibit and cared for by a few specialized keepers produced more total motile sperm/ejaculate than counterparts maintained on-exhibit (Koester et al., 2015). As a follow up to that study, here we examined the influence of the same environmental factors, that is, exhibit status and numbers of nearby conspecifics, on female ovarian and glucocorticoid profiles and concentrations. The hypotheses were that cheetahs managed (1) ‘off-exhibit’ or (2) away from other conspecifics, would exhibit more normal ovarian activity and have lower glucocorticoid metabolite concentrations than counterparts living in people-rich or cheetah-rich environments. To expand on observations of the single mother/daughter affiliative pair reported by Wielebnowski *et al.* (2002), we also assessed adult female siblings managed together since birth to test the hypothesis that long-term cohabitation of highly related females has no influence on gonadal or adrenal function.

2. Materials and methods

2.1. Animals and environmental conditions

Female cheetahs (n = 22) were housed at 10 institutions across the USA, all of which were accredited by the Association of Zoos & Aquariums (Table 1). Each cheetah was captive born and managed according to protocols and husbandry guidelines established by the Cheetah SSP (Ziegler-Meeks, 2009). The study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Study animals were 2 to 10 yr of age (Table 1; mean $\pm$ standard error of the mean [SEM], 6.1 $\pm$ 0.5 years), with individuals considered reproductively mature at 2 yr of age (Crosier et al., 2011). All facilities housed cheetahs in outdoor enclosures (ranging from 0.03 – 0.6 ha in size)

with access to a shelter. Diet included feeding a commercial meat product (beef or horse-based) at least 5 d/wk with occasional supplements of whole rabbit, bones (beef, horse, bison, venison), or organ meat (beef, horse, venison); water was available ad libitum (Crosier et al., 2016; Terrell et al., 2010).

Cheetahs were categorized as being housed either ‘on-exhibit’ (animals in visual contact with >50 different person visitors to the institution per month for ≥ 5 consecutive months) or ‘off-exhibit’. On-exhibit enclosures ranged from 0.03 to 0.3 ha in size, whereas off-exhibit enclosures were 0.10 to 0.60 ha. Cheetahs also were evaluated on the basis of the number of conspecifics housed in close proximity, that is the total number of other male or female cheetahs ≥ 2 yr of age in visual range of the test female (usually within 4 ha). All group-managed females were full siblings and living within that group since birth.

2. *Fecal collection and preparation*

Freshly-voided feces were collected a minimum of 4 to 5 d/wk for 12 to 24 wk/female. Approximately 50 g of each sample were placed in a labeled, plastic bag and stored at -20°C . When females were managed together in the same enclosure, a different non-digestible and harmless marker (i.e., glitter, birdseed, lentils, corn) was added to each food pan so that feces could be individually identified. Fecal samples ($n = 2,093$) were lyophilized (VirTis, 35L Ultra Super XL-70, Gardiner, NY), pulverized using a mallet, and stored at -20°C until processed further.

Steroid hormone metabolites were extracted from fecal samples using previously described methods (Graham and Brown, 1996). The efficiency of steroid extraction was

evaluated by adding radiolabeled hormone (^3H -progesterone or ^3H -cortisol; 4,000 – 8,000 dpm) to each fecal sample prior to boiling extraction. The overall mean ($\pm$ SEM) extraction efficiency for all samples was $75.2\% \pm 0.2\%$. Fecal extracts were diluted 1:20 in BSA-free phosphate buffer (2.2 M NaH_2PO_4 , 3.5 M Na_2HPO_4 , 0.3 M NaCl , H_2O ; pH, 7.0) for analysis by enzyme immunoassay (EIA). Fecal hormone data were expressed as $\mu\text{g/g}$ dry feces.

3. *Fecal estrogen metabolite analysis*

Estrogen metabolite concentrations in diluted fecal extracts were determined using an estradiol EIA validated for use in the cheetah (Crosier et al., 2011). This EIA relied on a polyclonal anti-estradiol antibody (R4972; C. Munro, University of California, Davis, CA) that cross-reacted with 17β -estradiol (100%), estrone (3.33%), and $< 0.01\%$ with estrone sulfate, progesterone, testosterone, cortisol, and corticosterone. Antibody (0.05 ml) was added to 96-well microtiter plates (Nunc-Immuno, Maxisorp; Fisher Scientific) and equilibrated for 12 to 48 h (4°C). Unbound antibody was removed with wash solution (1.5 M NaCl , 0.06 mM, 5.0 mL Tween 20 [Sigma-Aldrich, P1379]), and diluted samples (in duplicate) and standards (in triplicate) (0.02 ml; 97.5 – 25,000 pg/ml; 17β -estradiol; Sigma Diagnostics) were added to the plate. A peroxidase enzyme-conjugated 17β -estradiol (1:50,000; 0.05 ml; C. Munro) then was added to each well containing sample or standard and the plate incubated for 2 h (23°C) before unbound components were removed with wash solution. A chromagen solution (0.1 ml; 2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulphonic acid], ABTS) was added to each well and incubated ~ 40 min before optical densities were determined using a microplate reader (Dynex MRX, reading filter at 405 nm, reference filter at 540nm). Samples considered too

dilute (binding >80% of maximum) were assessed at a higher concentration (1:10), whereas those that were too concentrated (binding <20%) were run at a lower concentration (1:200).

Sensitivity of the estradiol EIA at maximum binding was 1.95 pg/well. The inter-assay coefficients of variation for two internal controls were 7.5% (mean binding, 27.5%) and 8.6% (mean binding, 70.7%), and intra-assay coefficient of variation was <10% (n = 98 assays). Serially-diluted, pooled fecal extracts expressed displacement curves parallel to those of the estradiol standard curve. Recovery of added estradiol to a fecal extract demonstrated significant recovery ($y = 1.3x - 4.0$, $r = 0.99$; $P < 0.05$).

4. *Fecal glucocorticoid metabolite analysis*

Glucocorticoid metabolite concentrations in diluted fecal extracts were determined using a cortisol EIA validated for the cheetah (Koester et al., 2015; Young et al., 2004). The polyclonal antibody used (R4866; C. Munro) was raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin. The antibody cross-reacted with cortisol (100%), prednisolone (9.9%), prednisone (6.3%), cortisone (5%), and <1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (Young et al., 2004). Microtiter plates were assessed as described above for estrogen analysis; diluted samples in duplicate, standards in triplicate (0.05 ml; 78 – 20,000 pg/ml; Sigma Diagnostics), and a peroxidase enzyme-conjugated cortisol tracer (1:20,000; 0.05 ml; C. Munro) were added to the plate. The latter was incubated for 1 h (23°C) before unbound components were removed, ABTS was added, and optical

density measured. Samples considered too dilute were evaluated at 1:10, and those that were too concentrated were assessed at 1:200.

Sensitivity of the assay at maximum binding was 3.9 pg/well. The inter-assay coefficients of variation for two internal controls were 10.6% (mean binding, 27.3%) and 6.2% (mean binding, 66.5%), and the intra-assay coefficient of variation was <10% (n = 113 assays). Serially-diluted, pooled fecal extracts expressed displacement curves parallel to the cortisol standard curve. Recovery of added cortisol standard to a fecal extract demonstrated significant recovery ($y = 0.9x - 18.0$, $r = 0.99$; $P < 0.05$).

5. *Statistical analysis*

The influence of the factors on- versus off-exhibit and number of cheetah conspecifics on hormonal metrics of single-housed females (n = 17) was determined using a mixed model ANOVA. Fixed factors included exhibit status (on- vs. off-exhibit to the public) and number of conspecifics (<10 cheetahs or ≥ 10 cheetahs of either sex held in nearby enclosures), while controlling for interaction between factors and holding female age as a covariate. The specific numerical cutoff for number of conspecifics was selected because sample size and distribution of females in the study did not provide sufficient statistical power for regression analysis of this factor on hormonal metrics. Therefore, a distinct, numerical category cutoff was selected (i.e., <10 or ≥ 10 conspecifics of either sex held in nearby enclosures at the same institution) to cluster females into two approximately even groups for analysis. To mitigate the potential of confounding effects of the two environmental factors examined, we conducted post-hoc evaluations using the non-parametric Kruskal-Wallis one-way analysis of variance test on

ranks. Thus, hormonal values were compared among females (1) on-exhibit in the presence of <10 conspecifics (n = 6 individuals), (2) off-exhibit in the presence of <10 conspecifics (n = 3), (3) on-exhibit in the presence of ≥ 10 conspecifics (n = 3), and (4) off-exhibit in the presence of ≥ 10 conspecifics (n = 5).

Differences in hormonal metrics between grouped females and those managed singly was tested using a Mann-Whitney U test (Grafen and Hails, 2002). Eleven females (n = 5 in two groups of 2 or 3 females each; n = 6 singly housed; Table 1) were included in this evaluation. Group-managed females were all maintained at on-exhibit facilities containing ≤ 10 conspecifics in nearby enclosures. Therefore, to avoid any confounding effects of exhibit status or number of other cheetahs in adjacent enclosures on variables in grouping analysis, singly-housed females used for comparison were also from on-exhibit institutions containing ≤ 10 conspecifics.

Prior to analysis, statistical outliers identified as those values outside of the interquartile range multiplied by a factor of 2.2, as defined by Hoaglin and Iglewicz (1987), were replaced with the nearest neighboring value (Sokal and Rohlf, 2012) for all hormonal variables (<1% of data). Normality and homoscedasticity were tested using an Anderson-Darling and a Levene's test, respectively (Grafen and Hails, 2002). When variables were not normally distributed and normality could not be achieved through outlier removal or transformation, nonparametric statistical tests were used (Grafen and Hails, 2002). Effects were considered significant at $P < 0.05$, and data are reported as mean $\pm$ SEM. All statistical procedures were completed using SAS software (version 9.2, Cary, NC).

Hormonal data for each female were summarized as follows: 1) an overall mean of all samples for the collection period; 2) a baseline of all values calculated using an iterative process, excluding values greater than the overall mean plus 1.5 standard deviations (SD) (Brown et al., 1996; Young et al., 2004); 3) a peak frequency that divided the total number of peak samples (see definitions below) by total number of samples collected for that female (expressed as a percent); 4) a mean peak amplitude that reflected average height of hormonal peaks above individual baseline values; and 5) anestrus period length calculated by dividing the total number of anestrus period days by total number of days within the sample collection period (expressed as a percent). An estrogen peak was the highest value within a group of samples >1.5 times baseline (Brown et al., 1996). Values greater than three times baseline were considered significant glucocorticoid peaks (Young et al., 2004). A follicular cycle was the interval between consecutive estrogen peaks that was equal to, or greater than, 4 days apart; any interval >30 days (i.e., twice the estimated ovarian cycle duration) was considered an anestrus period (Brown et al., 1996; Wielebnowski et al., 2002). Fecal samples were not collected daily from all females and, therefore, any missed estrogen peaks may result in an overestimation of anestrus periods. The pooling nature of fecal metabolites, however, as well as the fact that the prevalence of these instances of ovarian quiescence is similar between females sampled four to five times/week and those sampled daily, indicates little to no overestimation in the present study.

Linear associations between estrogen and glucocorticoid metabolite values in each female were evaluated using correlation analyses (Spearman's) (Grafen and Hails, 2002). Post-hoc comparison of hormonal metrics between anestrus periods and cyclic ovarian activity

periods within females ($n = 9$) was completed using a Wilcoxon signed-rank test (Grafen and Hails, 2002).

2. Results

1. *Influence of on- versus off-exhibit and number of conspecifics on female hormonal metrics*

All 22 female cheetahs demonstrated cycles of estrogen metabolite activity (229 total cycles peak to peak) that averaged 10.3 ± 0.9 days in length (range, 4 – 29 days). Periods of anestrus (range, 31 – 83 days) were observed in 41% of the females. Mean estrogen metabolite concentration for all females was 0.29 ± 0.01 $\mu\text{g/g}$ (range, 0.21 – 0.44 $\mu\text{g/g}$), with a baseline of 0.22 ± 0.01 $\mu\text{g/g}$ (range, 0.17 – 0.28 $\mu\text{g/g}$). Mean glucocorticoid concentration was 0.62 ± 0.07 $\mu\text{g/g}$ (range, 0.21 – 1.49 $\mu\text{g/g}$) with a baseline of 0.32 ± 0.04 $\mu\text{g/g}$ (range, 0.13 – 0.77 $\mu\text{g/g}$).

There were no differences ($P > 0.05$) in estrogen metabolite patterns (i.e., length and number of anestrus periods, frequency or amplitude of peaks) or mean concentrations between on- versus off-exhibit or ≥ 10 versus < 10 conspecific treatment groups (Table 2). Longitudinal glucocorticoid profiles revealed episodes of elevated excretion that were about three times above baseline occurring over 1- to 7-day intervals, but also were not related ($P > 0.05$) to the environmental factors evaluated here (Table 2). Correlations were observed between estrogen and glucocorticoid metabolite concentrations for both mean ($r_s = 0.53$; $P = 0.03$; Fig. 1) and baseline ($r_s = 0.51$; $y = 1.217x + 0.003$; $P = 0.04$) data.

There were no differences ($P > 0.05$) in hormonal metrics (for either excreted estrogen or glucocorticoid metabolite) among the four environmental conditions representing the two factors of interest (exhibit status and number of conspecifics present). There was a tendency ($P < 0.06$) for glucocorticoid peak frequency to be higher in females housed in on-exhibit facilities with ≥ 10 conspecifics present than counterparts held in off-exhibit facilities with ≥ 10 conspecifics (Fig. 2c).

Analysis of variables within individual females yielded, on average, a higher percentage ($P = 0.04$) of samples containing maximal glucocorticoid excretion coinciding with periods of cyclic ovarian activity ($69.7 \pm 6.6\%$) compared to during anestrus periods ($30.3 \pm 6.6\%$). Further analysis revealed increased glucocorticoid peak frequency (Fig. 3a; $P = 0.02$) and mean glucocorticoid concentration (Fig. 3b; $P = 0.02$) during intervals of ovarian estrogenic waves compared to periods of inactivity. This effect also was apparent in individual cheetah profiles (Fig. 4).

2. *Influence of single- versus group-management on female hormonal metrics*

There was no effect ($P > 0.05$) of maintaining female cheetahs in cohorts of two or three on excreted estrogen (see Fig. 5a for one pair) or glucocorticoid (see Fig. 5b for data on the sibling trio) profiles or average estrogen or glucocorticoid concentrations (Table 3). Both grouping types revealed brief waves of estrogenic cyclicity indicative of ovarian activity (Fig. 4 and 5a). There was no observed anestrus in any grouped female compared to the singletons that produced one to two periods (33 – 52 days in length) of ovarian quiescence during the 24

week fecal monitoring interval. There was no visually discernable, synchronous patterns of estrogenic activity among females maintained within the same group (Fig. 5a).

2. Discussion

For more than 2 decades, our laboratory has studied how intrinsic and extrinsic factors regulate reproductive success of cheetahs managed *ex situ* (Crosier et al., 2016; Donoghue et al., 1992; Pukazhenthhi et al., 2001; Wielebnowski and Brown, 1998). The cheetah is renowned for being challenging to breed and reproduce (Wildt et al., 2010), with some of this limitation attributable to environmental factors purportedly linked to ‘stress’ (Terio et al., 2004; Wells et al., 2004). Earlier anecdotal evidence, now supported by contemporary assessments of studbook data, make it clear that reproductive success is greater when cheetahs are managed in more spacious breeding centers away from intensive public exposure. Part of the benefit is related to simply having more cheetahs in one location and, thus, having more breeding options. For males, we have discovered significant physiological advantages in that most cheetahs in off-exhibit conditions produce more total sperm per ejaculate compared to zoo managed counterparts (Koester et al., 2015). Such a benefit also is incurred when care is provided by fewer, but consistently the same animal keepers (Koester et al., 2015). However, this increased sperm output on the basis of off-exhibit location and fewer care-givers is not mediated by any discernible relatedness to adrenal glucocorticoid patterns or concentrations (Koester et al., 2015). The inability in that recent work to find a relationship between adrenal and reproductive function in the cheetah is at odds with earlier assertions by those who have suggested that the species is particularly sensitive to stress in relation to social or

environmental factors, perhaps a cause for being a reproductive challenge in captivity (Jurke et al., 1997; Terio et al., 2004; Wells et al., 2004). Regardless, it is clear that the pairing of unfamiliar individuals, at least of females, can be disruptive to gonadal function (Wielebnowski et al., 2002). Given these observations, we examined the impact of on- versus off-exhibit management, number of conspecifics, and maintenance of singletons versus groups of siblings on both estrogen and glucocorticoid metabolite output. There was no impact of the type of management system (traditional zoo and fewer conspecifics versus a more spacious breeding center and many conspecifics) on estrogen or glucocorticoid concentrations or profiles in female cheetahs. Furthermore, managing familiar females (siblings) together was not disruptive or influential to ovarian or adrenal activity. For virtually all individuals, estrogen metabolite patterns were species-typical, with short, wave-like patterns or periods of baseline production. Glucocorticoids were excreted generally in a random fashion and with no evidence of species hypersensitivity in adrenal function. The sole relatedness between these two steroid hormones was a consistently higher excretion of glucocorticoids during the periods of elevated estrogenic activity.

Our earlier study of male cheetahs maintained in urban zoos or off-site, spacious breeding centers also found no evidence that patterns of excreted glucocorticoids were affected by housing type (Koester et al., 2015). Here we observed a similar lack of relationship between glucocorticoid concentrations and our analyzed housing metrics in adult females. Without data from wild animals, we cannot conclusively say if the levels observed in captive cheetahs are comparatively ‘high’ or ‘low’, but it is now clear from studies in both males and females that altered glucocorticoid excretion in response to conspecific numbers or exhibit status are

not apparent. However, an earlier study did find elevations in fecal glucocorticoids as cheetahs were moved into a zoo-exhibit environment and, conversely, declined when animals were transferred to off-site (non-public) facilities, suggesting there may be some unidentified components of captivity that do influence adrenal activity (Wells et al., 2004). We do not doubt that most animals, including the cheetah, undergo a clear increase in adrenal activity as a result of moving (or being moved) into a new environment. This phenomenon also has been well documented in recent years via fecal hormone monitoring for a wide variety of wildlife species (Christofoletti et al., 2010; Vick et al., 2012; Young et al., 2001). Change of home can cause clear elevations in excreted glucocorticoid concentrations that inevitably are followed by adaptation and a fall in adrenal hormonal metabolites over the course of days or weeks (Christofoletti et al., 2010; Nelson, 2005; Vick et al., 2012). Of course, adrenal acclimatization to any novelty is the result of the systemic effects of glucocorticoids that enable the organism's body to cope with and resist the original, or additional, stressors (Nelson, 2005). Our fecal monitoring protocol was both more intensive and longer in duration than the earlier investigation, but similar to a previous study that found no relationship between social housing-related ovarian suppression and glucocorticoid concentrations in female cheetahs (Wielebnowski et al., 2002). These authors concluded that fecal glucocorticoid concentrations might not be the best way to assess response to perceived stressors in that circumstance. It also was noteworthy that these adrenal steroid metabolites were excreted in a highly variable fashion, and it was not unusual for concentrations to differ by at least three-fold within a 24 h period. Such a finding led to two important conclusions, the first being that adrenal function as measured in this carnivore was dynamic even when

measured via fecal monitoring that naturally provides a ‘pooling effect’ of metabolites (Young et al., 2004). More importantly, for fecal glucocorticoid measures to be useful for understanding physiological function, serial, frequent sampling was required. With sufficient longitudinal data, as generated in the present study, then it was possible to be comfortable with interpretation and observed effects or trends. Although post-hoc analysis revealed a tendency for females in on-exhibit facilities holding ≥ 10 conspecifics to exhibit a higher glucocorticoid peak frequency than off-exhibit facilities with similarly high cheetah numbers, the incidence of peaks was not different between these exhibition types in the presence of < 10 conspecifics. The tenuous nature of this trend also was supported by the lack of a similar relationship when assessing mean glucocorticoid concentrations. Thus, we suspect that this one trend towards potential significance probably resulted from some institutional idiosyncrasy that was unidentified (and uncontrollable) in the present study. One such observed relationship was the elevated glucocorticoid concentrations measured during times of highest estrogen excretion, periods assumed associated with heightened follicular activity. The cheetah does not routinely demonstrate overt behavioral signs of sexual receptivity, as observed in many other felid species (Leyhausen and Tonkin, 1979; Michael, 1961; Umapathy et al., 2007). It is common to see coincidental increases in physical activities, including pacing, rolling, urine spraying, and vocalizing during suspected periods of estrus (Wielebnowski and Brown, 1998). Although measuring behavioral activities in our cheetah cohort was not a part of our investigation, it made sense that there was increased glucocorticoid production during times of elevated excreted estrogens. For example, domestic cat studies have demonstrated that increased estrogen effects on the brain reflect increased

physical activity (Michael, 1962). While mediating physiological responses to perceived stressors, glucocorticoids also function as metabolic regulators during predictable events such as maximal sexual activity, including mating. Evidence for this phenomenon, as generated by fecal hormonal monitoring, is available for diverse birds (Crossin et al., 2013), the giant panda (Kersey et al., 2010), gray wolf (Eggermann et al., 2013), and Canada lynx (Fanson et al., 2012). In each of these examples there are higher excreted glucocorticoid concentrations during times of breeding for one or both sexes. These elevations may be related to the increased energetic demands of reproduction (e.g., soliciting the mate) or stresses associated with the actual interactions of the male and female (Crossin et al., 2013; Eggermann et al., 2013). Regardless, clearly the cheetah fits within this paradigm of more glucocorticoid excretion during periods of heightened estrogen production. Such findings suggested the presence of probably important nuances to the adrenal-reproductive system relationship that depended on duration of elevated glucocorticoids. For example, others have contended that chronically elevated adrenal activity must be a disadvantage to reproductive success and well-being in felids (Mellen, 1991; Moreira et al., 2007; Wells et al., 2004). In contrast, our observations indicated that short-term increases in glucocorticoids may have been supporting normal physiological functioning during peak estrual activity in the cheetah.

Managing cheetahs in the presence of many near-by conspecifics had no influence on either estrogen or glucocorticoid concentrations or patterns, thereby revealing no tendency for animal numbers to be causing ovarian cycle disruptions or physiological stress. These observations aligned with an earlier study of adult male cheetahs that demonstrated no differences in fecal androgen and glucocorticoid concentrations as well as ejaculate quality

and diverse behaviors, regardless of being managed in the presence of fewer or more male conspecifics (Koester et al., 2015). Therefore, there seems to be no reason for concern that reproductive success in *ex situ* collections is limited by having too many cheetahs of either sex in the same location. This lack of sensitivity in the captive environment to number of conspecifics has some relevance to what has been measured in wild cheetahs. For example, it is known that free-ranging cheetahs in eastern Africa have comparative small territories due to protected area boundaries (Caro, 1994; Gottelli et al., 2007). As a result, individual cheetahs (both males and females) often overlap range, and yet reproductive success is no different from that of counterparts in southern Africa that have naturally larger home areas with fewer geographic impediments (Caro, 1994; Gottelli et al., 2007). Taken together, this factor of cheetah density or number (*in situ* or *ex situ*) has limited impact on reproductive metrics evaluated to date and, specifically from our studies (Koester et al., 2015; present observations), no ramifications on the main hormones produced by the gonads or adrenal glands.

Wielebnowski *et al.* (2002) have reported reproductive suppression (sometimes mutually) in unfamiliar females that are placed together, with reproductive activity recommencing rather quickly once pairs are separated, even into adjacent enclosures separated by a chain-link fence. These same investigators, using fecal estrogen monitoring, found typical patterns of ovarian cyclicity only in a bonded mother-daughter pair that was showing strong affiliative behaviors (Wielebnowski et al., 2002). Kinoshita and colleagues (2011) also reported an instance of normative excreted estrogen activity in two female cheetahs maintained together long-term in the absence of inter-animal aggression. In our study of more cheetahs, we too

found no significant differences between females managed as singletons or chronically in small sibling groups. All individuals expressed cheetah typical estrogen patterns, and singleton and grouped females had similar excreted concentrations of estrogen or glucocorticoid metabolites. While no formal behavioral monitoring was conducted, anecdotally we observed no inter-animal aggression during the course of the investigation. Therefore, there was no evidence that this small, same sex, familial groupings of female cheetahs influenced either ovarian estrogenic or adrenal glucocorticoid activity.

In summary, there was no detectable influence of female cheetahs living in people-dense, urban zoos versus in off-site breeding centers on the ability of the ovaries or adrenal glands, respectively, to produce species-typical estrogen and glucocorticoid concentrations and patterns. Having these females managed in the presence of high numbers of conspecifics within the same facility or even multiple individuals within the same enclosure also had no effect on these same metrics. There also was no evidence of physiological stress on the basis of glucocorticoid metabolite monitoring. Cheetahs, regardless of ‘environmental’ treatment, produced similar fecal concentrations and patterns. At the same time, the highest glucocorticoid amounts coincided with bouts of the most excreted estrogen, probably reflecting more physical activities associated with likely (but mostly silent) estrous periods. These findings have practical implications. For example, it has become conventional wisdom that cheetahs are highly vulnerable to environmental stressors, particularly those imposed by captive environments. However, we found no such evidence in glucocorticoid metabolite concentrations, either in the present study for females or a recent publication for males (Koester et al., 2015). Yet, clearly most reproductive success *ex situ* for this species is

occurring in large-size breeding centers rather than smaller urban zoos. This ‘space factor’ has exerted at least one physiological advantage for the male cheetahs, the production of more total sperm per ejaculate (Koester et al., 2015). We can only conclude that we (and others) have been targeting the wrong limiting factor for successful reproduction. Rather than focusing on chronic physiological stress measured by fecal glucocorticoid metabolites, cheetah researchers may want to consider better understanding the advantages of breeding centers where clearly most of the matings, pregnancies, and cub production now are occurring (Marker, 2012; Wildt et al., 2012). For example, future areas of emphasis could include the benefits of the sheer additional space of these large facilities for more exercise, more naturalistic stimuli, and more conspecifics for provoking reproductive interest and mate opportunities. Finally, finding that long-term groupings of female cheetahs had no impact on ovarian or adrenal endocrine function suggested that there was no detrimental effect from managing cheetahs in small cohorts when space was limiting. There is some anecdotal evidence that such females exposed to a male will breed in the presence of other females, although it is prudent to remove the male once the targeted female is suspected of being pregnant (Ziegler-Meeks, 2009). The impact of grouping on developmental biology of juvenile and pubertal cheetahs remains to be studied, especially the benefits or disadvantages of living with conspecifics on subsequent ability to develop normative behaviors to reproduce successfully.

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Table 1

Demographic and management information for female cheetahs (n = 22) used in this study.

Facility	Studbook number	Age (yr)	Conspecific grouping	Management
Birmingham Zoo ^a	5757 ^b	3.2	<10	Group
Birmingham Zoo ^a	5759 ^b	3.2	<10	Group
Chehaw Wild Animal Park ^a	6532 ^b	4.9	<10	Group
Chehaw Wild Animal Park ^a	6533 ^b	4.9	<10	Group
Chehaw Wild Animal Park ^a	6534 ^b	4.9	<10	Group
Dickerson Park Zoo ^a	5756 ^b	8.4	<10	Singly
Dickerson Park Zoo ^a	5758 ^b	8.4	<10	Singly
White Oak Conservation Center	5992	3.8	≥10	Singly
White Oak Conservation Center	4177	7.2	≥10	Singly
White Oak Conservation Center	4494	6.2	≥10	Singly
Philadelphia Zoo ^a	5674 ^b	5.1	<10	Singly
Fort Worth Zoo ^a	3961 ^b	7.6	<10	Singly
Mesker Park Zoo ^a	6480 ^b	7.0	<10	Singly
Smithsonian Conservation Biology Institute	5816	6.6	≥10	Singly
Smithsonian Conservation Biology Institute	6163	9.0	≥10	Singly
Smithsonian Conservation Biology Institute	4453	7.4	<10	Singly
Smithsonian Conservation Biology Institute	4568	8.0	<10	Singly
Smithsonian Conservation Biology Institute	6592	2.1	<10	Singly
Smithsonian National Zoological Park ^a	6339 ^b	3.7	<10	Singly
Fossil Rim Wildlife Center ^a	3527	10.2	≥10	Singly
Fossil Rim Wildlife Center ^a	3979	7.3	≥10	Singly
Fossil Rim Wildlife Center ^a	4261	5.3	≥10	Singly

^aIndicates facility was considered on-exhibit for analysis

^bIndicates inclusion in cohabitation analysis (n = 6 singly-managed females for comparison)

Table 2

Anestrous period and fecal estrogen and glucocorticoid data (mean $\pm$ SEM) for female cheetahs managed (1) on- versus off-exhibit or (2) in the presence of 10 or more or fewer than 10 conspecifics (n = 17).

	On-exhibit	Off-exhibit
No. of females	9	8
Anestrous period length (% of total collection days)	22.7 ± 7.7	19.6 ± 8.1
Estrogen concentration:		
Mean (µg/g)	0.30 ± 0.03	0.26 ± 0.01
Baseline (µg/g)	0.23 ± 0.01	0.21 ± 0.01
Peak frequency (%)	10.2 ± 1.2	11.4 ± 2.4
Mean peak amplitude (µg/g)	0.30 ± 0.06	0.21 ± 0.01
Glucocorticoid concentration:		
Mean (µg/g)	0.52 ± 0.10	0.45 ± 0.08
Baseline (µg/g)	0.24 ± 0.03	0.30 ± 0.08
Peak frequency (%)	16.7 ± 5.7	14.8 ± 3.2
Mean peak amplitude (µg/g)	0.98 ± 0.14	1.11 ± 0.22
	≥10 Conspecifics	<10 Conspecifics
No. of females	8	9
Anestrous period length (% of total collection days)	28.8 ± 7.2	14.4 ± 7.6
Estrogen concentration:		
Mean (µg/g)	0.27 ± 0.01	0.29 ± 0.03
Baseline (µg/g)	0.22 ± 0.01	0.22 ± 0.01
Peak frequency (%)	9.6 ± 2.4	11.8 ± 1.2
Mean peak amplitude (µg/g)	0.22 ± 0.02	0.29 ± 0.06
Glucocorticoid concentration:		
Mean (µg/g)	0.57 ± 0.15	0.52 ± 0.10
Baseline (µg/g)	0.31 ± 0.08	0.23 ± 0.02
Peak frequency (%)	13.7 ± 2.2	17.7 ± 6.0
Mean peak amplitude (µg/g)	1.02 ± 0.22	1.05 ± 0.14

Table 3

Anestrous period and estrogen and glucocorticoid data ($\pm$ SEM) for female cheetahs that were group- compared to singly-managed.

Metric	Group-managed (n = 5)	Singly-managed (n = 6)
Anestrous period length (% of total collection days)	none	13.4 $\pm$ 9.2
Estrogen metabolite concentration ($\mu\text{g/g}$)		
Mean	0.27 $\pm$ 0.01	0.35 $\pm$ 0.04
Baseline	0.22 $\pm$ 0.02	0.23 $\pm$ 0.02
Peak frequency (%)	12.8 $\pm$ 1.6	12.3 $\pm$ 1.4
Mean peak amplitude ($\mu\text{g/g}$)	0.22 $\pm$ 0.05	0.46 $\pm$ 0.09
Glucocorticoid metabolite concentration ($\mu\text{g/g}$)		
Mean	0.61 $\pm$ 0.17	0.73 $\pm$ 0.14
Baseline	0.38 $\pm$ 0.09	0.33 $\pm$ 0.05
Peak frequency (%)	6.6 $\pm$ 2.1	19.3 $\pm$ 8.1
Mean peak amplitude ($\mu\text{g/g}$)	1.04 $\pm$ 0.28	1.53 $\pm$ 0.19

Figure captions

Fig. 1. Correlation between mean fecal estrogen and glucocorticoid metabolite concentrations for female cheetahs ($n = 17$).

Fig. 2. Estrogen and glucocorticoid metabolite metrics for female cheetahs living in one of four environmental groups reflecting public exhibit status and number of conspecifics present. Anestrous period length (c) was represented by the percentage of the sample collection period where estrogen metabolite production was negligible and non-cyclic. Estrogen and glucocorticoid peak frequency (c) was the percentage of total sample collection days where hormone values were of peak concentration. The asterisk indicates the only tendency for a difference ($P < 0.06$) between two groups.

Fig. 3. a) Frequency of glucocorticoid peaks in fecal samples from cheetahs ($n = 9$) during anestrual versus cyclic ovarian periods. Peak frequency was the number of samples containing a hormone concentration more than three times the calculated baseline divided by the total number of samples collected for that female profile, expressed as a percentage value. b) Average glucocorticoid and estrogen metabolite concentrations in fecal samples from cheetahs ($n = 9$) during anestrual versus cyclic ovarian periods. Within each panel, values with different superscripts differed ($P < 0.05$).

Fig. 4. Fecal estrogen and glucocorticoid metabolite profiles in four representative cheetahs (a – d) that experienced periodic anestrus. Solid horizontal lines represented the calculated peak cutoff values for each hormone where samples yielding hormone concentrations above this value were considered ‘peaks’. An estrogen peak was the highest value within a group of samples >1.5 times baseline, and

values greater than three times baseline were considered glucocorticoid peaks. Each asterisk indicated a peak.

Fig. 5. Longitudinal fecal estrogen (a) and glucocorticoid (b) metabolite profiles for two (a) and three (b) group-managed female cheetahs. The horizontal lines indicate the calculated baseline for each corresponding female, with each asterisk indicating a peak.

Figure1

Figure2

Figure3

Figure 5