Non-invasive assessment of adrenocortical activity as a measure of stress in leopards (*Panthera pardus*)

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Abstract

Leopards (*Panthera pardus*) are listed in the IUCN Red list as vulnerable, primarily due to habitat loss, natural prey base depletion and exploitation caused by various anthropogenic activities. Although protected areas are important for leopard conservation, the majority of suitable leopard habitat lies beyond protected area boundaries exposing individuals to different environmental, physiological and psychosocial stressors. This study aimed to examine the suitability of five different enzyme immunoassays (EIAs) for monitoring adrenocortical function in the leopard based on faecal glucocorticoid metabolite (fGCM) analysis. After performing an adrenocorticotrophic hormone (ACTH) stimulation test and investigating the stability of fGCM post-defaecation, faeces from free-ranging leopards in a peri-urban and a conservation area was collected to investigate the potential impact of habitat variability on glucocorticoid output. An EIA measuring fGCM with a 5α-3β-11β-diol structure performed best, demonstrating a ~200 - 330% increase in fGCM concentrations approximately 40 h post-ACTH administration. Concentrations of fGCM remained quite stable for up to six-days post defaecation and showed a maximum increase of 8% and a maximum decrease of 9%. Although not significantly different (MWRS T = 86, n = 16, p = 0.920), overall median fGCM concentrations were 68% higher in individuals utilising the peri-urban area compared to leopards utilising the conservation area. The ranges of fGCM concentrations between sites however were similar. Individual median fGCM concentrations differed distinctly between free-ranging males and females, possibly linked to female reproductive status. The established method can now assist in addressing some of the issues facing local wildlife managers, conservationists and researchers tackling

various aspects related to leopard conservation and management under different land use practices.

Key words: ACTH Challenge, cortisol, faecal glucocorticoid metabolites, fGCM, fGCM stability post-defaecation, habitat variability.

INTRODUCTION

Historically, leopard (*Panthera pardus* Linnaeus, 1758) distribution extended to all continents with the exception of Australia and Antarctica (Macdonald and Loveridge 2010), but current information suggests that leopards persist globally in only 25% of their previous range (Jacobson et al. 2016). Province-wide population studies in South Africa reinforce this trend demonstrating severe declines, (as much as 40% in some areas) in leopard densities over the last four years (Swanepoel et al. 2016; Williams et al. 2017). Reasons for this include an anthropogenic-driven loss of suitable habitat, human-wildlife conflict, depletion of natural prey bases and exploitation of leopards through trophy hunting and hunting for traditional practices (Swanepoel et al. 2014), but given that this elusive predator is difficult to study in the wild, detailed population estimates are lacking (Stein and Hayssen 2013; Swanepoel et al. 2016; Williams et al. 2017). Catholic use of habitat and versatility as a generalist predator allows leopards to successfully occupy any habitat, including altered natural habitats and settled environments that offer sufficient cover for ambush hunting and which supports sufficient numbers of adequately-sized prey species (Hayward et al. 2006; Swanepoel et al. 2014). Thus, the majority of suitable leopard habitat lies beyond conservation area boundaries on private or community-owned land (Martin and De Meulenaar 1988; Boitani 1999; Marker and Dickman 2005) and leopards moving across a land-use matrix may find themselves exposed to different environmental, physiological and psychosocial stressors. The ability of individuals to cope with acute stress or the potentially harmful effects of chronic stress that can compromise reproductive ability, cause muscle atrophy and suppress the immune system (Möstl and Palme 2002) have not yet been investigated in this species.

Stress is defined as any stimulus that threatens or is perceived to threaten the homeostasis of an individual (Selye 1936). The series of adaptive mechanisms aimed at protecting an individual and restoring homeostasis is known as the stress response (Sheriff et al. 2011). When a stressor is perceived, the hypothalamic-pituitary-adrenocortical axis and the sympatho-adrenomedullary system are activated, which results in an increase in glucocorticoid and catecholamine secretion (Sapolsky 2002; Palme et al. 2005). As part of the acute stress

response, catecholamines are secreted within fractions of a second and increase the availability of glucose from energy stores (Palme et al. 2005; Sheriff et al. 2011). Glucocorticoid responses may be adaptive in the short-term; however, prolonged elevation of glucocorticoid levels due to chronic stress can be disruptive (Möstl and Palme 2002). Concentrations of glucocorticoids or their metabolites determined from biological matrices such as blood, saliva, urine or faeces, can therefore act as reliable indicators for levels of disturbance experienced by an individual (Palme et al. 2005; Sheriff et al. 2011; Möstl 2014). Non-invasive monitoring of responses to various stressors, using faeces as hormone matrix has gained popularity, particularly for the monitoring of elusive, free-roaming species (Ganswindt et al. 2012; Kersey and Dehnhard 2014). However, due to species-specific differences in steroid metabolism, any technique to monitor faecal glucocorticoid metabolite (fGCM) concentrations in a species for the first time must be thoroughly validated to ensure that the measured compounds reflect circulating concentrations of biologically active target agents (Touma and Palme 2005; Schwarzenberger 2007; Ganswindt et al. 2012). An adrenocorticotropic-hormone (ACTH) challenge test that pharmacologically induces physiological changes in circulating glucocorticoid levels is the most widely accepted physiological validation method (Touma and Palme 2005). The aim of our study was thus to examine the suitability of available enzyme immunoassays (EIAs) for non-invasive monitoring of fGCM concentrations from free-ranging leopards utilizing different habitats. More specifically, we a) determined stress-related physiological responses in leopard faeces by performing an adrenocorticotrophic hormone stimulation test (ACTH challenge), b) investigated the effect of bacterial metabolism and environmental factors on the stability of fGCM concentrations post-defaecation, and c) compared fGCM concentrations from free-ranging leopards utilizing a conservation area and a peri-urban area.

METHODS

Study animals

For the first part of the study, two adult leopards (one male 65 kg; one female 45 kg) housed in separate but adjacent enclosures at Predator World, North West Province, South Africa, were monitored. Individuals were fed 6–8 kg portions of venison, beef, chicken, donkey or horsemeat, three times a week and the diet was supplemented with an assortment of powdered vitamins and minerals as necessary. Each enclosure provided adequate fresh water, resting sites, play items and covered "night houses" and individuals received clear visual, olfactory and auditory stimulus from other large predators kept nearby including Bengal tiger

(*Panthera tigris tigris* Linnaeus, 1758), lion (*Panthera leo* Linnaeus, 1758) and spotted hyaena (*Crocuta crocuta* Erxleben, 1777).

For the second part of the study, fresh faecal material was collected from two adult leopards (one male 56 kg; one female 46 kg) housed together in the same enclosure at Lory Park Zoo, Midrand, Gauteng Province, South Africa. The enclosure contained sand-cement substrate low-lying vegetation, adequate shade, additional raised resting areas and unrestricted access to "night houses". Fresh water points situated in the open-air enclosure could be accessed at any time and the enclosure was cleaned daily. The leopards at this facility were fed 2–4 kg portions of horsemeat five days a week. Powdered vitamins and mineral additives supplemented their diet as required.

For the third part of the study, data from free-ranging leopards at the Hoedspruit Wildlife Estate (HWE), Maruleng Municipality, Limpopo Province, South Africa and at MalaMala Private Game Reserve (MMGR), Mpumalanga Province, South Africa, were collected. Nine individually recognized free-ranging male and female leopards utilize the 6.9 km^2 peri-urban HWE, which has a road density of 6.23 km of road/km^2, is fenced on all boundaries and accommodates approximately 1 100 full-time residents in housing with associated infrastructure and amenities. Approximately 27 (females 16; males 11) individually recognized leopards utilize the 125 km^2 conservation area of MMGR, which has a road density of 4.12 km of road/km^2. The property lies open to the Sabi Sand Game Reserve (650 km^2) to the north, south and west, and the Kruger National Park (22 000 km^2) to the east and accommodates approximately 209 beds associated with commercial tourist operations. Both field sites experience summer rainfall with generally frost-free winters. Altitude (250 – 700 m above sea level), mean annual rainfall (450 – 700 mm) and mean monthly temperatures, (min -1°C and max 39°C) for June and January respectively (Skukuza) are similar at both study sites (Fischer et al. 2014; Viljoen and Moller 2015). The study was conducted with the approval of the University of Pretoria Animal Use and Care Committee (Reference V016-16).

ACTH challenge test

The two animals at Predator World were separated on the first day of data collection and housed in adjacent enclosures for the 14-day duration of the ACTH challenge experiment. Although visual, auditory and olfactory contact was maintained between individuals through the adjoining fence for the entire monitoring period, separation was categorised as a potential stressor and thus formed part of the biological validation for the EIAs. For seven days pre- and seven days post-ACTH administration, individuals were observed from 07h00 to 18h30 each day

and all faecal samples voided during this time were collected within 10 min post-defaecation, immediately frozen and stored at -20°C until analysis. As enclosures could not be accessed at night, all samples voided overnight (18h30 - 07h00) were collected the following morning at 07h30 and stored frozen at -20°C until analysis. On day eight, each individual received an intramuscular injection of synthetic ACTH (2.3 - 2.5 IU/kg, Synacthen® Depot, Novartis) delivered through 30 mm un-collared treatment needles by a registered veterinarian, using a short-barrel Dan-Inject® (Dan-Inject® International, Denmark) remote dart gun operating under CO_2 pressure. Notably, the immobilization and translocation of a neighbouring Bengal tiger took place on day four of the experiment.

Stability of faecal glucocorticoid metabolite concentration post defaecation

To determine the effects of bacterial metabolism and environmental factors on the stability of immunoreactive fGCM concentrations post-defaecation, six fresh faecal samples were collected from the individuals housed at the Lory Park Zoo. The material was thoroughly mixed using a hand-held stick blender, and then divided into 42 subsamples of which half were stored outside under full sun and the other half under full shade conditions. Subsequently, three sub-samples were collected from the full sun and full shade treatments at time = 0 h as a control, and thereafter at intervals of 1 h, 6 h, 12 h, 2 days, 3 days and 6 days respectively. Collected material was immediately frozen and stored at -20°C until analysis.

Faecal sample collection from free-ranging leopards

All faecal material at MMGR study site was collected opportunistically by safari guides viewing animals for tourism purposes. After a defaecation event, samples were collected, put on ice and frozen within eight hours until analysis. Faecal material collected from free-ranging individuals at HWE was located opportunistically and frozen within three hours of collection. At HWE, a 15 km stretch of gravel road network in the undeveloped "green zone" was checked for leopard tracks and faeces on foot each morning between 05h00 and 09h00. When fresh leopard tracks were located, spoor was followed to locate fresh (< 10 h) scat. Samples were individually labelled and kept cool in ice-pack containers until they could be stored frozen at -20°C. All samples remained frozen until they reached the Endocrine Research Laboratory, University of Pretoria for further processing.

Steroid extraction and analysis

Frozen samples were lyophilised, pulverised and sieved through a wire-mesh strainer to remove any undigested material and between 0.10 - 0.11 g of faecal powder from each sample was then mixed with 3 ml of 80% ethanol, vortexed for 15 min and centrifuged for 10 min at 1500 g

for steroid extraction. Supernatant (1.5 ml) was decanted and stored at -20°C until analysis (Ganswindt et al. 2002). Due to the absence of information regarding the presence and relative abundance of faecal glucocorticoid metabolites (fGCM) specific to the leopard, faecal steroid extracts resulting from the ACTH challenge were measured for immunoreactive fGCM concentrations using five different enzyme immunoassays (EIAs) namely: "(i) Cortisol, (ii) 11-oxoaetiocholanolone I (detecting 11,17 dioxoandrostanes), (iii) 11-oxoaetiocholanolone II (detecting fGCMs with a 5β - 3α -ol-11-one structure), (iv) Corticosterone and (v) 5α -pregnane-3β ,11β ,21-triol-20-one (detecting fGCMs with a 5α -3β -11β -diol structure). Detailed assay characteristics, including full descriptions of the assay components and cross reactivity's have been provided for the 11-oxoaetiocholanolone I, cortisol and corticosterone EIAs by Palme and Möstl (1997), 11-oxoaetiocholanolone II EIA by Möstl et al. (2002) and for the 5α-pregnane-3β,11β,21-triol-20-one EIA by Touma et al. (2003). Subsequently, the sample sub-set related to the investigation of fGCM concentration stability post-defaecation was analysed using only the 11-oxoaetiocholanolone I and the 5α-pregnane-3β,11β,21-triol-20-one EIAs. Finally, samples collected from free-ranging leopards at the MMGR and HWE study sites were analysed using only the 5α -pregnane-3β ,11β ,21-triol-20-one EIA.

Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve in the 11-oxoaetiocholanolone I and the 5α-pregnane-3β,11β,21-triol-20-one EIAs (relative variation (%) of the slope of respective trend lines < 4%). The sensitivities of the EIAs used were 0.6 ng/g dry weight (DW) (Cortisol, 11-oxoaetiocholanolone I and 11-oxoaetiocholanolone II EIA), 1.8 ng/g DW (Corticosterone EIA) and 2.4 ng/g (5α -pregnane-3β ,11β ,21-triol-20-one EIA) respectively. Intra-assay coefficients of variation of low- and high-value quality controls were 4.8% and 5.8% (Cortisol EIA), 4.0% and 4.8% (11-oxoaetiocholanolone I EIA), 5.3% and 5.8% (11-oxoaetiocholanolone II EIA), 5.7% and 5.9% (Corticosterone EIA), and 4.6% and 5.7% (5α -pregnane-3β ,11β ,21-triol-20-one EIA) respectively. Inter-assay coefficients of variation of low- and high-value quality controls were only determined for 11-oxoaetiocholanolone I EIA (6.5% and 11.6%) and 5α -pregnane-3β ,11β ,21-triol-20-one EIA (6.2% and 14.4%). All steroid concentrations are given as µg/g faecal dry weight. All fGCM extractions and subsequent hormone analyses were performed at the Endocrine Research Laboratory, University of Pretoria, South Africa as described previously (Ganswindt et al. 2002).

Data analysis

Due to the occurrence of biological stressors prior to ACTH administration, assay-dependent individual baseline fGCM concentrations were determined by using the median fGCM concentration of the four samples collected from 64.5 h post-ACTH administration onwards. Subsequently, assay-dependent fGCM concentration for each sample was expressed as a percentage (%) using individual baseline fGCM concentrations as 100%. To determine responses to physiological or biological stressors, the fGCM concentration (%) of the first voided sample after the occurrence of each respective stressor was compared with respective individual baseline fGCM concentrations. Stability of fGCM concentration post-defaecation was calculated using the median fGCM concentration determined at time = 0 h (100% initial concentration), to calculate differences in fGCM concentration for each sample. Subsequently, mean differences in fGCM concentration for each triplicate sample set was calculated. The Chi–square test was used to determine statistically significant differences between the two storage treatments (full sun and full shade) tested. Descriptive statistics (overall median and range) and a Mann-Whitney Rank Sum test (MWRS) were used to compare fGCM concentrations determined from individuals at MMGR and HWE sites and for comparison of fGCM concentrations of males and females at and between study sites. At HWE, collected material was assigned (80-90% confidence by CyberTracker level III accredited tracker) to either male or female individuals using track and sign interpretation (Liebenberg 2000) where possible (n = 17 out of n = 37). Subsequently, HWE sex-assigned sample sets were compared using a Mann–Whitney U-test (MWU) after testing for normality with a Shapiro–Wilk test. Statistical analyses were performed using the software programme Statistica (v.13) and statistical significance was assumed when $P < 0.05$. Data are presented as means ± SD, medians and ranges where applicable. SigmaPlot (v.10) was used for all graphical presentation of data.

RESULTS

ACTH challenge test and biological validation

Using a set minimum increase of 100% as a target, the 11-oxoaetiocholanolone II EIA performed best for the male (334% increase from 1.81 μg/g DW baseline concentration), but performed inadequately for the female (33% increase from 3.88 μg/g DW baseline concentration). The 11-oxoaetiocholanolone I EIA performed adequately, showing an increase of 173% from baseline concentration (0.48 μg/g DW) in the male and 130% increase from baseline concentration (0.37 μg/g DW) in the female. Overall, the 5α-pregnane-3β,11β,21-

triol-20-one EIA performed best in both individuals; with a 331% increase from baseline concentration (0.39 μg/g DW) reflected in the male, and a 203% increase from baseline concentration (0.29 μg/g DW) reflected in the female (Table 1 and Figure 1). Peak fGCM concentrations were present in the first faecal sample post-ACTH administration (40.7 h) across four of the five assays except for the Cortisol EIA, which measured a peak at 66 hours post-injection in the male (Table 1). Using the 5α-pregnane-3β,11β,21-triol-20-one EIA, fGCM concentrations took longer to return to baseline in the male 87.0 ± 33.9 h (mean ± SD; range 64.7 - 112.7 h) than in the female 65.3 ± 0.53 h (mean ± SD; range 65.0 - 65.8 h). In the first sample voided post-separation, peak fGCM concentrations increased by 54.8% from baseline in the male (47.4 h) and by 30.5% from baseline in the female (25.0 h). Immobilization and translocation of a neighbouring Bengal tiger resulted in an increase of 170% (after 22.9 h) and 141.3% (after 25.3 h) from baseline fGCM concentrations in the male and female respectively (Figure 1).

Stability of fGCM concentration post-defaecation

Under full-sun conditions the 11-oxoaetiocholanolone I EIA showed a maximum decrease in fGCM concentration of 10% within the first hour and a maximum increase in fGCM concentration of 7% after 48 h. Under full-shade conditions, the 11-oxoaetiocholanolone I EIA showed a maximum decrease in fGCM concentration of 20% after 48 h and a maximum increase of 8% after 72 h (Figure 2). In contrast, under full-sun conditions, the 5α-pregnane-3β,11β,21-triol-20-one EIA showed a maximum decrease of 6% after 72 h and maximum increase of 6% after 144 h. Under full-shade conditions, the 5α-pregnane-3β,11β,21-triol-20-one EIA measured a maximum decrease of 9% after 6 h and a maximum increase of 8% after 72 h (Figure 2). Distribution of fGCM concentration across sampling subsets did not differ between sun and shade conditions for the 11-oxoaetiocholanolone I EIA (X^2 p = 0.61, df = 1) or the 5α-pregnane-3β,11β,21-triol-20-one EIA (X^2 p = 0.63, df = 1). Overall, the 5α-pregnane-3β,11β,21-triol-20-one EIA performed best for measuring differences in fGCM concentrations and thus was used for further analysis of samples from free-ranging animals.

Comparison of FGCM concentrations at different study sites

In total seven samples were collected from the MMGR study site and 17 samples were collected from the HWE study site. Although overall median fGCM concentrations differed between the conservation area (MMGR: 0.79 μg/g DW) and the peri-urban area (HWE: 1.16 μg/g DW) study sites by 68%, the groups were not significantly different (MWRS T = 86, n = 16, p = 0.920). The range showed pronounced variation at both sites, with fGCM concentrations

Adult Male	Cortisol	11-oxo-aetiocholanol-one I	Corticosterone	11-oxo-aetiocholanol-one II	5α-pregnane-3β,11β,21-triol-20-one
Baseline fGCM concentration (μg/ g DW)	**0.18**	**0.48**	**0.94**	**1.81**	**0.29**
Peak fGCM concentration – Separation (μg/ g DW)	0.08	0.81	1.18	4.86	0.45
Hours after stressor to reach peak concentration	47.35	47.35	47.35	47.35	47.35
Response (%)	-55.16	67.54	24.94	168.12	54.75
Peak fGCM concentration – Translocation (μg/ g DW)	0.09	0.84	0.80	5.73	0.79
Hours after stressor to reach peak concentration	22.80	22.80	22.80	64.55	22.80
Response (%)	-51.36	75.44	-14.51	216.33	170.96
Peak fGCM concentration - ACTH Challenge Test (μg/ g DW)	0.20	1.11	1.79	7.85	1.25
Hours after injection to reach peak concentration	65.7	40.66	40.66	40.66	40.65
Response (%)	13.04	129.91	90.30	333.55	331.19

Adult Female	Cortisol	11-oxo-aetiocholanol-one I	Corticosterone	11-oxo-aetiocholanol-one II	5α-pregnane-3β,11β,21-triol-20-one
Baseline fGCM concentration (μg/ g DW)	**0.12**	**0.37**	**1.50**	**3.88**	**0.39**
Peak fGCM concentration – Separation (μg/ g DW)	0.15	0.44	1.09	5.06	0.51
Hours after stressor to reach peak concentration	24.35	24.35	24.35	24.35	24.35
Response (%)	23.15	21.20	- 27.82	30.31	30.46
Peak fGCM concentration – Translocation (μg/ g DW)	0.20	0.94	2.38	8.22	0.95
Hours after stressor to reach peak concentration	25.33	25.33	21	21	25.33
Response (%)	62.69	155.34	26.95	111.93	141.34
Peak fGCM concentration - ACTH Challenge Test (μg/ g DW)	0.24	1.00	2.69	5.66	1.19
Hours after injection to reach peak concentration	40.75	40.75	40.75	40.75	40.75
Response (%)	94.22	172.95	78.53	33.25	203.05

TABLE 1.
Baseline and peak fGCM concentrations, % increase and time taken to reach peak fGCM concentration for two adult leopards at Predator World, South Africa after perceiving three stressors (physical separation, immobilisation and translocation of a neighbouring Bengal tiger (Panthera tigris tigris) and ACTH administration for each of the five EIAs tested.
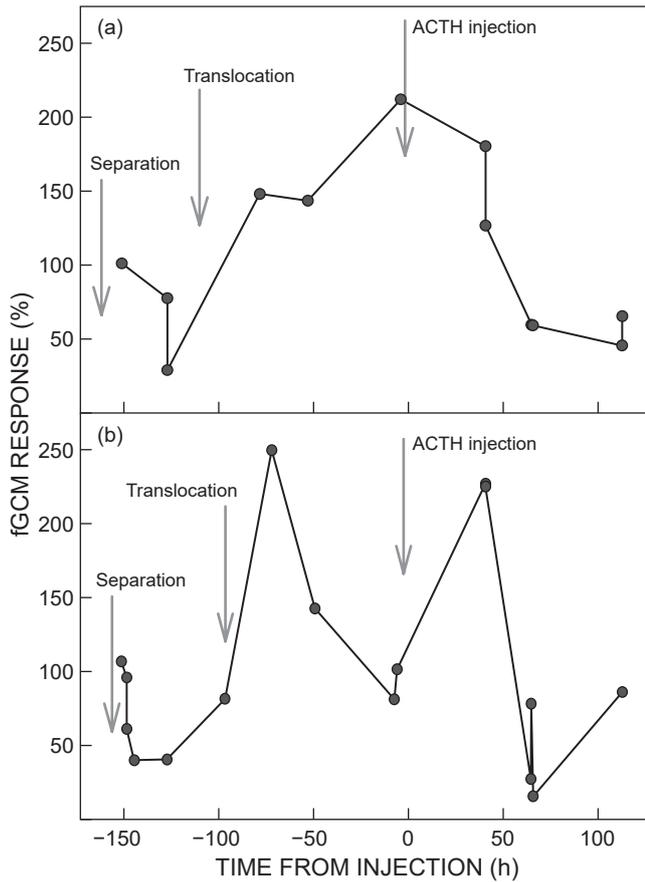
Figure 1: Longitudinal profiles of immunoreactive faecal gluco-corticoid metabolite (fGCM) concentration percentage increase determined using the most stable enzyme immunoassay (5α- pregnane-3β,11β,21-triol-20-one) for the adult female (a) and adult male (b) leopards housed at Predator World following separa-tion, immobilisation–translocation of a neighbouring Bengal tiger (*Panthera tigris tigris*), and adrenocorticotrophic hormone (ACTH) administration. Vertical grey arrows indicate potential stress events
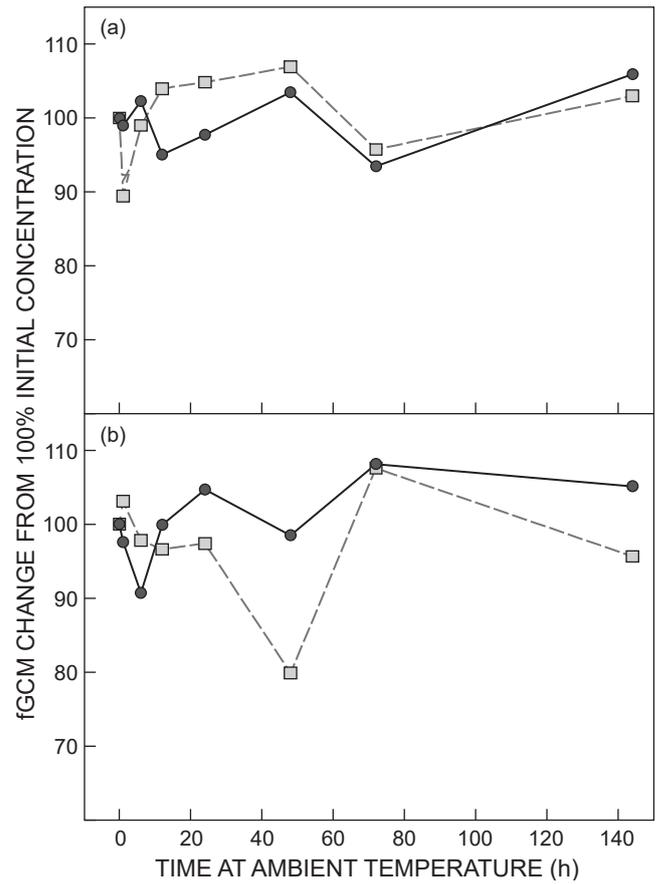
Figure 2: Changes in faecal glucocorticoid metabolite (fGCM) concentrations post-defaecation for the 11-oxoaetiocholanolone I enzyme immunoassay (grey broken line) and 5α-pregnane-3β,11β,21-triol-20-one concentrations (black solid line) over time for faeces stored in full sun (a) and full shade (b) conditions
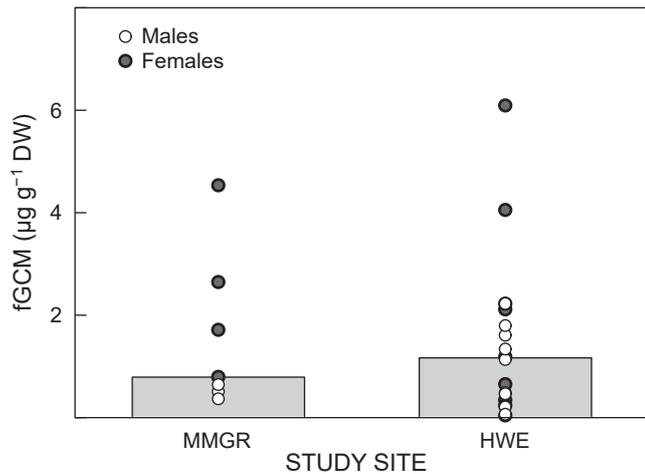
Figure 3: Box-bar plot for individual faecal glucocorticoid metabolite (fGCM) concentrations collected from males and females at the MalaMala Private Game Reserve (MMGR) and Hoedspruit Wildlife Estate (HWE) study sites. At the MMGR study site, samples were collected from known individuals and at HWE samples were assigned to different sexes using track and sign interpretation. Circles in each column represent fGCM concentrations of sampled individuals at each study site, and the bar in each column represents the median value of fGCM concentrations measured at each site

measured in the MMGR animals (0.36 – 4.53 μg/g DW) being similar to those measured in the HWE animals (0.02 – 6.09 μg/g DW). Overall median fGCM concentrations were 50% higher in MMGR females (n = 4) compared to males. Interestingly, two females were known to be pregnant or rearing young, and showed respective fGCM concentrations of 428% and 806% higher than the overall mean steroid concentrations measured in the males (n = 3) from the same site. At the HWE study site, samples assigned to males (n = 8) or females (n = 9) were not statistically different (MWU = - 0.82, n = 17, p = 0.41). The range of fGCM concentrations allocated to females (0.04 – 6.09 μg/g DW) was again greater than the range allocated to males (0.06 – 1.79 μg/g DW) (Figure 3). However, if the two samples from females with the highest fGCM concentrations are excluded, the range of fGCM concentrations allocated to females (0.04 – 2.22 μg/g DW) is similar to the fGCM concentrations measured in the males. Baseline concentration of the captive male (0.29 μg/g DW) and captive female (0.39 μg/g DW) at Predator World fall within the lower range of fGCM concentrations determined for free-ranging males and females respectively.

DISCUSSION

Of the five enzyme immunoassays tested in this study, the cortisol and corticosterone EIAs performed poorly in both sexes, presumably because the majority of biologically active steroids are heavily metabolised before excretion and thus, specificity of the respective antibodies used are suboptimal (Möstl and Palme 2002). The three remaining group-specific EIAs that were tested revealed suitable results for at least one (11-oxoaetiocholanolone II) or both (11-oxoaetiocholanolone I and 5α-pregnane-3β,11β,21-triol-20-one) sexes for detecting physiologically and biologically meaningful changes in stress-related differences in fGCM concentrations in the leopard. Sex-related quantitative differences in fGCM concentrations in response to a stressor are not uncommon (Ludwig et al. 2013) as, irrespective of the principal glucocorticoid present in the blood, steroid hormones may be metabolised differently between sexes of the same species, resulting in sex-specific differences in metabolite composition within the faeces (Touma et al. 2003, Touma et al. 2004). Apart from the mechanisms involved, a number of species including African buffalo (*Syncerus caffer* Sparrman, 1779; Ganswindt et al. 2012), Nile crocodile (*Crocodylus niloticus* Laurenti, 1768; Ganswindt et al. 2014), spotted hyaena (van Meter et al. 2009) and bat-eared fox (*Otocyon megalotis* Desmarest, 1822; Le Roux et al. 2016) have been described to show sex-related differences in fGCM concentrations in response to an ACTH challenge. Further, respective sex-related differences in stress-induced

glucocorticoid differences have been noted in closely related members of the cat family including the domestic cat (*Felis catus* Linnaeus, 1758; Young et al. 2004), clouded leopard (*Neofelis nebulosa* Griffith, 1821; Wielebnowski et al. 2002; Young et al. 2004), jaguar (*Panthera onca* Linnaeus, 1758; Conforti et al. 2011), cheetah (*Acinonyx jubatus* Schreber, 1775; Ludwig et al. 2013), lion (Creel et al. 2013) and tiger (Bhattacharjee et al. 2015). Of course, the differences in stress-related fGCM alterations found between the two animals, could also reflect the variation in individual coping ability within specific captive situations (Wielebnowski et al. 2002; Ludwig et al. 2013; Chosy et al. 2014).

Immunoreactive fGCM concentrations can decrease or increase in relation to assay specificity depending on the fGCM stability post-defaecation (Lexen et al. 2008). A decrease in fGCM concentrations post-defaecation has been noted in sheep (*Ovis aries* Linnaeus, 1758; Lexen et al. 2008), brown hyaena (*Parahyaena brunnea* Thunberg, 1820; Hulsman et al. 2010) and Nile crocodile (Ganswindt et al. 2014), while marked increases have been noted in domestic cattle (*Bos taurus* Linnaeus, 1758), horses (*Equus ferus* Linnaeus, 1758), pigs (*Sus scrofus domesticus* Linnaeus, 1758) (Möstl et al. 1999) and sheep (Lexen et al. 2008). In contrast, Meza-Cruz et al. (2014) demonstrated that for the jaguar, changes in immunoreactive fGCM concentrations post-defaecation were not significant, with fGCM concentrations remaining relatively stable for up to five days in both the wet and dry season. In the current study, immunoreactive fGCM concentrations measured with the 5α-pregnane-3β,11β,21-triol-20-one EIA showed overall less variation and greater stability for up to 6 days post-defaecation compared to respective steroid concentrations determined with the 11-oxoaetiocholanolone I EIA. A reliable measurement of fGCM concentrations in leopard faeces that is up to six days old allows a more feasible approach for monitoring free-roaming individuals, for example, designated transects could be checked and cleared every five to six days without having to precisely age faecal samples. However, potential seasonal variability in the stability of fGCM concentrations in the leopard should be further assessed in future studies.

Overall median fGCM concentrations were 68.1% higher in the peri-urban HWE study site than they were in the conservation area of MMGR. The data should however be interpreted with caution, as samples from the MMGR study site were collected from only seven (four females and three males) of apparently 27 individual animals using the property and might therefore not be a true representation of fGCM concentrations of animals within that population. Further, samples collected from the seven animals utilising the HWE study site could not be assigned to specific individuals. Despite these limitations, our initial findings suggest that

factors at the HWE study site, contributed to overall higher individual fGCM concentrations as well as greater variability than those determined in the conservation area of the MMGR study site. This is in line with studies conducted on elk (*Cervis elephas* Linnaeus 1758), wolves (*Canis lupus* Linnaeus, 1758; Creel et al. 2002), spotted hyaena (Van Meter et al. 2009), lion (Creel et al. 2013), and tiger (Bhattacharjee et al. 2015), which demonstrate that anthropogenic disturbance can result in elevated fGCM concentrations. Although small data sets were obtained from the MMGR and HWE study sites during this study, higher variability driven by some extreme values was found in measured fGCM concentrations of females at both sites. This pattern matches findings in female South African Lesser Galagos (*Galago moholi* Smith, 1836; Scheun 2015) and female white rhinoceros (*Ceratotherium simum simum Burchell, 1817;* Badenhorst et al. 2016) that demonstrate a potential link between alterations in pregnancy-related progestagen and fGCM concentrations.

Information from known female individuals at the MMGR study site provided some insights and possible explanation for these sex-related differences. Two of the four monitored females at the MMGR study site showed particularly high fGCM values. Anecdotal and observation data related to sightings, associations and denning behaviour gathered by the MalaMala safari guides during daily drives, showed that the two MMGR females demonstrating comparatively high fGCM values were pregnant and approaching parturition or rearing young. Interestingly, the highest fGCM concentrations determined from the HWE sample set were also assigned to females. Further studies specifically designed to assess the link between adrenocortical activity and the phases of reproduction in both captive and free-ranging leopards would however be necessary to better interpret the patterns detected in the current study. Factors such as age, temperature, food availability, life history stages, levels of habituation and others (McNab 2002; Conforti et al. 2011; Naidenko et al. 2011, Goymann 2012) could also contribute to the variability in fGCM concentrations measured in these individuals. Further studies investigating the effects of these variables on fGCM concentrations are therefore necessary.

The ability to reliably assess adrenocortical function in male and female leopards using the 5α-pregnane-3β,11β,21-triol-20-one EIA which performed best overall, provides a useful non-invasive tool to further examine both intrinsic (cyclicity, pregnancy, lactation) and extrinsic (persecution in farming communities, levels of tourist activity, human-predator conflict situations) factors potentially acting as environmental stressors, and thus facilitate the development of locally applicable leopard management and conservation strategies for free-ranging and captive individuals.

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