Non-invasive monitoring of glucocorticoid metabolite concentrations in native Indian, as well as captive and re-wilded tigers in South Africa

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Highlights

- Proximity to humans may increase fGCM levels and negatively impact their welfare.
- Pregnant tigers have higher fGCM levels than males and non-pregnant females.
- Neither season nor sex impacts tiger fGCM concentrations or welfare.

Abstract

Over the last century, wild tiger (*Panthera tigris*) numbers have declined from over 100 000 individuals to fewer than 4 000, with animals now confined to less than 5% of their historic range due to habitat loss, persecution, inadequate management, and poaching. In contrast, 15 000–20 000 tigers are estimated to be housed in captivity, experiencing conditions vastly different than their wild counterparts. A total of 280 tigers are currently held at 44 different facilities within South Africa, including zoos, semi-captive 're-wilded' populations, and pets; these animals provide a unique opportunity to measure the impact of extrinsic factors, found in exotic habitats, on the adrenocortical activity of tigers. By monitoring and comparing stress-related faecal glucocorticoid metabolite (fGCM) concentrations of tigers housed at different locations, and free ranging tigers in natural tiger reserves, this project aimed to get a better understanding of the impact of extrinsic factors on adrenocortical function as a measure of stress. The results of this study showed no significant difference in fGCM concentrations between captive, re-wilded, and free-ranging tigers with the exception of one site. Furthermore, factors such as sex and season were not significant drivers of fGCM concentrations. One study group had elevated fGCM concentrations, showing population variation in the stress response. This indicates that populations are able to cope with exotic environments, however, as population-specific differences in the stress response exist, we suggest management protocols be created for each population. This study offered the unique opportunity to see how well tigers are faring outside of their native range and if

having re-wilded tigers in exotic locations is a potential welfare-acceptable management option for tiger conservation globally.

Keywords: Adrenocortical stress response; Conservation physiology; Cortisol Faecal glucocorticoid metabolites; Non-invasive hormone monitoring

1. Introduction

Apex predators play important roles in ecosystem functioning (Ritchie et al., 2012) and can help protect against disease transmission (Wilmers et al., 2006), the effects of climate change (Pongsiri et al., 2009), and invasive species (Wallach et al., 2010). The loss of these top predators can unbalance an ecosystem and cause a trophic cascade (Wallach et al., 2015). Although important in their ecosystems, constant persecution has resulted in large carnivores being among the most threatened species globally (Woodroffe, 2000).

Tigers (*Panthera tigris*) have undergone severe range contractions and are at risk of extinction (Kenney et al., 2014). Due to habitat loss, persecution, poaching, and inadequate management and protection practices, tiger numbers have decreased from 100 000 to just below 4 000, and occupy <5% of their historic range (Dinerstein et al., 2007, Damania et al., 2008, Luo et al., 2008, Kenney et al., 2014, Duangchantrasiri et al., 2015, Wolf and Ripple, 2017).

Tigers under human care number between 15 000 to 20 000 individuals worldwide, and thus are five to seven times more abundant than their wild counterparts (Luo et al., 2008). Captive tigers experience environmental conditions vastly different from those encountered by their free-ranging counterparts (Szokalski et al., 2012). Firstly, compared to the size of their natural home ranges, which range from seven to 1000 km^2, captive tigers are often confined to small enclosures (Breton and Barrot, 2014). Furthermore, many captive tigers face major problems with in- and out-breeding practices (Nyhus et al., 2010). The captive environment can be detrimental and alter behaviour as has been seen in tigers; Pitsko (2003) showed that tigers kept in more stimulating and natural environments display more natural behaviours, including exploring, and less unnatural, stereotyped behaviours, such as pacing. Tigers within novel environments frequently encounter a range of stressors, and thus often display stereotypical behaviours; behavioural alterations that help animals cope with the perceived stressors of their unnatural surroundings and are generally considered an indication of stress in captive animals (Carlstead, 1996, Quirke et al., 2012, Mason et al., 2013).

The original definition of stress, proposed by Selye (1936), is “the nonspecific response of the body to any demand”; that is, any disruption to the body’s homeostasis and the response of the animal. When a noxious stimulus is perceived by an individual, there are both behavioural and physiological responses, such as heightened alertness, the suppression of reproductive and feeding processes, increased heart rate and blood pressure, and the redirection of energy reserves (Stratakis and Chrousos, 1995, Vingerhoets and Perski, 2000). Physiologically, two main response axes are activated; firstly, the sympatho-adrenal axis which causes the release of catecholamines from the adrenal medulla in the fight-or-flight response (Palme et al., 2005, Arun, 2006) and secondly, the

hypothalamic–pituitary-adrenal (HPA) axis, which results in the secretion of glucocorticoids (GCs) (Dedovic et al., 2009). Short-term elevation in GC concentrations is often advantageous as it can boost immune responses and mobilize energy reserves (Dhabhar, 2009). However, long-term elevation of GC concentrations can be deleterious as it can negatively affect reproduction, survival, and fitness of an animal (Tilbrook et al., 2000, Glaser and Kiecolt-Glaser, 2005, Dhabhar, 2009). As such, GC concentrations can act as a robust proxy of adrenocortical function and animal health (Khansari et al., 1990, Pottinger, 1999, Lupien et al., 2009).

Glucocorticoids can be quantified via invasive or non-invasive techniques (Ganswindt et al., 2012). Invasive techniques, such as hormone quantification in blood, have advantages such as real-time hormone concentrations. However, they have major drawbacks such as the restraint of animals that can lead to heightened GC concentrations and thus biased results (Hodges et al., 2010). These drawbacks have led to a burgeoning interest in exploring the use of alternative matrices to study adrenocortical function; as a result, non-invasive approaches have become the cornerstone of hormone monitoring in wildlife (Hodges et al., 2010). Non-invasive faecal sampling in particular, has advantages including the ease of collection and the ability to collect multiple samples from one individual without requiring direct human-animal interaction or impacting individual behaviour (Touma and Palme, 2005). Additionally, as faecal samples integrate fGCM concentrations over a period of time, they are less likely to show fluctuations due to stochastic events (Touma and Palme, 2005). However, non-invasive techniques always need to be validated for a new species, either biologically using a presumed stressor such as transportation, or physiologically using a synthetic hormone (Palme, 2019). When the two methods are used in parallel, the most understanding is gained (Touma and Palme, 2005).

Tigers within the captive environment offer a unique source to supplement sparse wild populations and ensure species survival. As such, facilities where tigers are kept should focus on enhancing management practices, specifically, removing unnecessary stressors within their immediate environment. Non-invasive endocrine monitoring in tigers offers a perfect tool to assess adrenocortical activity in the species, as previously shown (Naidenko et al., 2011, Narayan et al., 2013, Bhattacharjee et al., 2015, Malviya et al., 2018, Tyagi et al., 2019). This can benefit tigers by improving their welfare, fitness, survival, and reproduction, leading to the potential to reintroduce more tigers into the wild, a management programme that has already had some success (Bhattacharjee et al., 2015, Sarkar et al., 2016). The aim of this study was to quantify faecal glucocorticoid metabolites (fGCMs) as a measure of physiological stress in relation to season and environmental variables in tigers occurring in three different environments: the wild (within nature reserves in their native range in India), re-wilded (free roaming tigers in a reserve in South Africa that hunt for themselves and have no man-made shelter), and in captivity (tigers kept in relatively limited space in zoo-like indoor-outdoor enclosures).

2. Materials and methods

2.1. Study animals and sample collection

Faecal samples were collected from tigers in eight locations; five in South Africa (Fig. 1) and three in India (Fig. 2). The five locations in South Africa included the National Zoological Gardens (NZG), Lory Park Zoo (LP), Predator World (PW), a pet tiger (hereafter referred to as House), and Tiger Canyon (TC). The three locations in India were Nehru Zoological Park (NZP), Kanha Tiger Reserve (KTR), and Bandhavgarh Tiger Reserve (BTR). Related information on climate (climate-data.org) and tiger location is given in Table 1. In South Africa, the specific subspecies are not known, but many are suspected to be hybrids of Bengal (*P.t. tigris*) and Siberian tigers (*P.t. altaica*), while the Indian tigers we collected samples from were all Bengal tigers. Faecal samples for fGCM monitoring were collected seasonally in South Africa (winter: June – July 2019, and summer: December 2019 – January 2020). Due to logistical challenges, seasonal sampling could not be conducted at the three Indian sites, and samples were collected from the NZP between January and March of 2019, and in September of 2015 for KTR and BTR. No samples were kept in a freezer for longer than eight months prior to drying.

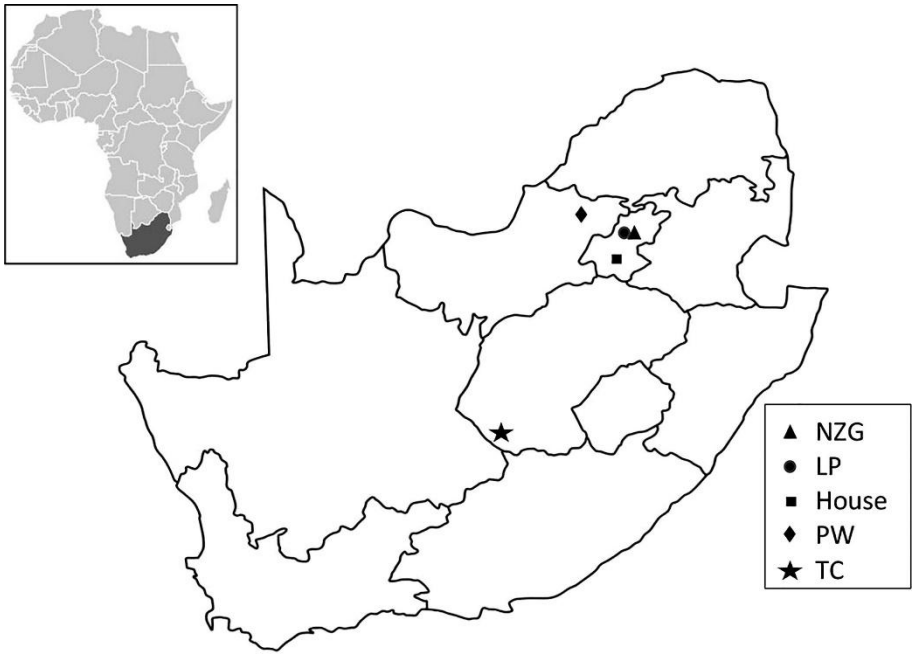

Fig. 1. A map showing the location of the study sites within South Africa. The insert shows where South Africa is located on the African continent.

Table 1. Climate and sample data for all study sites. Rainfall is the average annual rainfall and temperature range is the average temperature from the coldest month to the average temperature for the warmest month. The coordinate for House is a general coordinate for the city of Johannesburg as exact coordinates are confidential. Visitors is the number of visitors in 2019. The number of samples reflects the number that were collected (W – Winter, S – Summer) and analyzed and individual collection refers to whether there could be individual identification of the faecal samples that were deposited.

	Location							
	NZG	LP	PW	House	TC	NZP	KTR	BTR
Environment	Captive	Captive	Captive	Captive	Re-wilded	Captive	Wild	Wild
Province	Gauteng	Gauteng	Northwest	Gauteng	Free State	Telangana	Madhya Pradesh	Madhya Pradesh
Coordinates	25.7361S, 28.1902E	26.0098S, 28.1522E	25.3553S, 27.1636E	26.2041S, 28.0473E	30.2514S, 25.0394E	17.3507 N, 78.4513E	22.345 N, 80.6115E	23.4158 N, 80.5743E
Rainfall (mm)	697	697	663	790	391	766	1277	1277
Temp range (°C)	11.0–22.4	11.0–22.4	11.5–23.7	10.0–19.9	6.4–23.0	21.5–33.3	16.7–34.1	16.7–34.1
Visitors	340,386	48,251	5876	NA	655	NA	NA	NA
No. tigers	2F 1 M	2F 2 M	5F 3 M	1 M	~18 (var.)	11F 11 M	60 total	60 total
Samples per season	6 W 2 S	109 W 70 S	21 W 21 S	4 W 2 S	39 W 24 S	39 total	36 total	20 total
Individual collection	Yes	Yes	No	Yes	No	Yes	No	No
Born at that facility	Yes	Yes	No	No	Yes	Yes	Yes	Yes
Public view	Yes	2Yes 2No	Yes	No	Variable	Variable	Variable	Variable

Fig. 2. A map showing the location of the study sites within India. The insert shows where India is located on the Asian continent.

At the NZG, three tigers were kept in two enclosures (a male–female pair and a single female). At night, animals were kept in separate night rooms; all faecal samples excreted in these rooms were collected the following morning. Lory Park has four tigers, kept in two enclosures. A male–female pair is housed in an enclosure open for public viewing; the remaining male–female pair is housed in a separate enclosure which is only accessible by LP staff members. After defecation, the tigers were moved into a holding pen to allow for safe sample collection. Predator World has eight tigers, housed in pairs or groups of three, and samples were collected in a similar fashion to LP. Samples collected from the pet tiger occurred after defaecation when he was moved into his holding pen.

Tigers located at TC were all born on-site and kept in three large fenced off areas averaging 1200 ha where they have to fend for themselves; these animals are defined as "re-wilded". This study population fluctuated with births, deaths and translocations of young tigers old enough to disperse; a new area was also opened for tiger habitation during the study. Fresh faecal samples were collected opportunistically by driving around the areas, especially on boundary lines. Samples were positively identified as tiger samples using the information

provided by Pagett (2007) and due to the fact that they were the only carnivores of that size in their enclosures.

During the study period Nehru Zoological Park had 22 tigers. Samples were collected every morning when the cages were cleaned and the tigers moved into a sectioned-off area. Kanha Tiger Reserve (940 km^2, ~60 tigers) and BTR (1598 km^2, ~60 tigers) are both located in the state of Madhya Pradesh. Fresh samples were collected during forest surveys of roads and trails during non-tourism periods (September). Sample age at BTR and KTR was morphologically identified by outline shape, moisture content, and smell (Tyagi et al., 2019). Samples were collected evenly across the reserve to eliminate any potential sampling bias. All samples were collected using gloves and placed into small, sealable sample bags and immediately put on ice until they could be frozen at −20 °C. Gloves were replaced after every use to avoid cross-contamination of samples.

This study was approved by the animal ethics committee of the University of Pretoria (NAS019/2019) as well as the South African National Biodiversity Institute (SANBI) NZG Research Ethics and Scientific Committee (P19/11). Research permission to collect tiger faecal samples from the Indian tiger reserves were also obtained (from the Principal Chief Conservator of Forests, Madhya Pradesh letter Reference No. 7616, dated 12 October 2014).

2.2. Alteration in fGCM concentrations post-defecation

To determine the stability of fGCM concentrations post-defecation, six additionally collected faecal samples from two LP tigers were pooled, well-mixed, subdivided into 24 subsamples, and placed outside in partial sun under a slatted roof. Over six days, the samples were exposed to the elements. The average ambient temperature was 17 °C, with no cloud cover or rain during this period. Three subsamples were taken immediately after sub-dividing (0 h) and three additional subsamples were taken at time intervals of 1 h, 6 h, 12 h, 48 h, 96 h, and 144 h as described by Webster et al. (2018), and immediately frozen at −20 °C, a treatment that has been shown to have little effect on fGCM concentrations, even if frozen for years (Hunt and Wasser, 2003).

2.3. Steroid extraction and enzyme-immunoassay (EIA) analyses

The extraction process of the South African and Indian samples was identical apart from the initial drying process. Frozen faeces from South African tigers were lyophilized (as described by Heistermann et al., 2006), while respective samples from Indian tigers were dried in a hot air oven at 60 °C for two - three days (as described by Naidenko et al., 2019). Subsequently, individual samples were pulverized and sieved through a mesh strainer to remove undigested material such as bones and/or plant material (Fieß et al. 1999). Following this, 0.100 – 0.110 g of dried faecal powder was extracted with 3 ml 80% ethanol. After vortexing for 15 min, the suspension was centrifuged for 10 min at 1500 g (Webster et al. 2019). The supernatants were then transferred into microcentrifuge tubes and stored at − 20 °C until analysis.

Steroid extracts were analysed using an 11-oxoetiocholanolone II EIA (detecting fGCMs with a 5β-3α-ol-11-one structure) as described by Möstl et al. (2002) following EIA validation (see below). The sensitivity for the assay, calculated at 90% binding, was 0.18 ng/g DW, while the coefficients of variance, determined by repeated measurements of high- and low-value quality controls, were 6.1% and 8.7% (intra-assay) and 13.3% and 15.5% (inter-assay). Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve (relative variation of the slope of the trendlines < 3%) (Fig. 3). The same quality controls, steroid solution utilizing the commercially available 11-oxoaetiocholanolone (5β-androstane-3α-ol-11, 17-dione) from Steraloids Inc. (product number: A3460-000), were used on both the South African and Indian sample extracts to ensure comparable results.

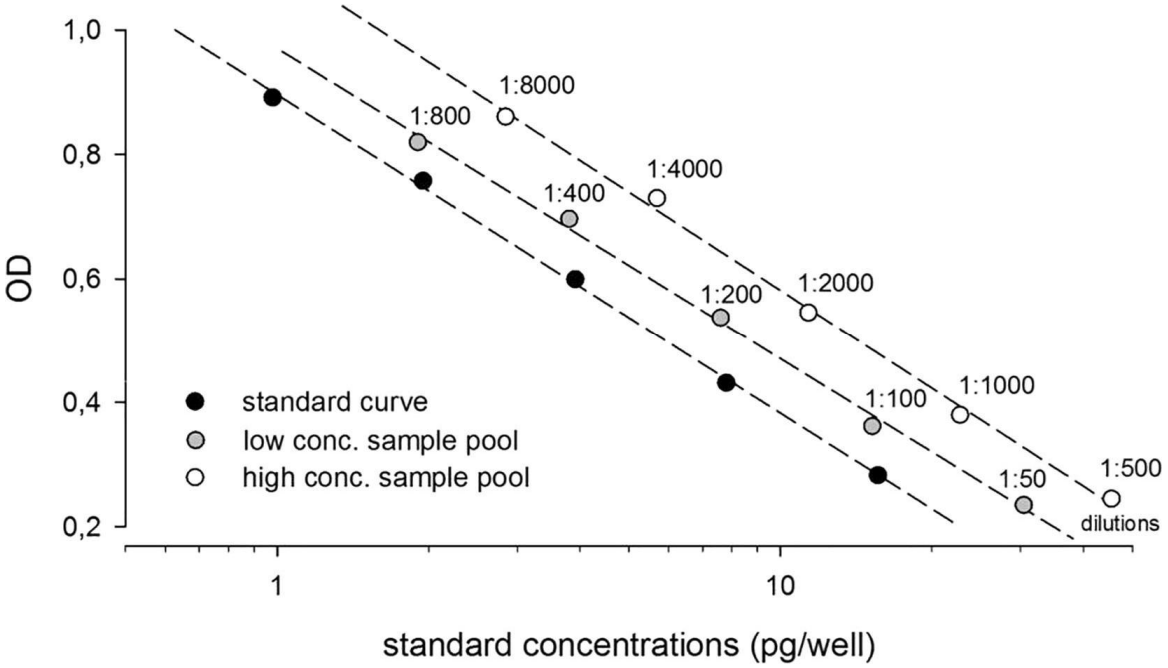

Fig. 3. Parallelism test for serial dilutions of low and high concentration sample pools for the selected 11-oxoetiocholanolone II enzyme-immunoassay.

All analyses of South African samples were performed at the Endocrine Research Laboratory, University of Pretoria, and all Indian samples were analysed at the Laboratory for the Conservation of Endangered Species, Centre for Cellular and Molecular Biology, as described by Ganswindt et al. (2002).

2.4. Biological validation of enzyme-immunoassays (EIA)

To evaluate EIA suitability, faecal extracts from a male and female Bengal tiger at the NZG were utilized. All faecal samples deposited 5 days prior to (total n = 10) and 10 days post (total n = 31) a translocation event were collected and analysed using five different EIAs: i) cortisol; ii) 11-oxoetiocholanolone I (detecting 11,17 dioxoandrostanes); iii) 11-oxoetiocholanolone II (detecting fGCMs with a 5β-3α-ol-11-one structure); iv) corticosterone; v) (iii) 5α-pregnane-3β,11β,21-triol-20-one (measuring 3β,11β-diol-cortisol metabolites). The respective assay characteristics including antibody cross-reactivities for the additional four tested EIAs are described by Palme and Möstl (1997) for the cortisol, 11-

oxoetiocholanolone I, and corticosterone EIAs, and by Touma et al. (2003) for 5α-pregnane-3β,11β,21-triol-20-one EIA. The sensitivities for these four assays were 0.18 ng/g (cortisol and 11-oxoetiocholanolone I), 0.54 ng/g (corticosterone) and 0.72 ng/g (5α-pregnane-3β,11β,21-triol-20-one). The intra-assays coefficients of variance were 9.5% and 11.4% (cortisol), 5.5% and 6.2% (11-oxoetiocholanolone I), 4.5% and 7.9% (corticosterone), and 4.9% and 6.3% (5α-pregnane-3β,11β,21-triol-20-one), while the inter-assay coefficients of variance were 10.8% and 18.4% (cortisol), 13.9% and 17.7% (11-oxoetiocholanolone I), 6.9% and 14.5% (corticosterone), and 12.2% and 16.6% (5α-pregnane-3β,11β,21-triol-20-one).

The study was approved by the NZG Research Ethics and Scientific Committee (P14-09).

2.5. Statistics

All analyses were conducted in R (R Core Team, 2017). All data were tested for normality using a Shapiro-Wilk goodness of fit test; only the post-defecation alteration data were parametric.

For the biological validation of the EIAs, median fGCM concentrations prior to the translocation event were used as the baseline for each animal and set as 0%. A repeated measure analysis of variance (ANOVA) was run to test for alterations in fGCM concentrations post-defaecation. The different time points at which the subsamples were taken were considered separate treatments. A general linear model (GLM) was conducted to determine the effect of season on fGCM concentrations; both rainfall and visitor number were found to be dependent on season and thus were not run as separate factors. Kruskal Wallis analyses of variance were run to quantify the effects of location and sex on fGCM concentrations. Additionally, a post-hoc pairwise Wilcoxon rank sum test was conducted to determine which locations had significant differences in fGCM concentrations, as well as to determine whether there was any difference between fGCM concentrations of on-display and off-display tigers at LP. Individual differences in fGCM concentrations were also tested at LP with a Kruskal Wallis analysis of variance followed by a Mann Whitney U post-hoc analysis. Differences in fGCM concentrations between individuals at other locations could not be conducted, as for most sites, only the sex of the tiger that provided the sample was supplied and not the individual identity. A Kruskal-Wallis analysis of variance was used to compare sexes in the different locations, with post-hoc Mann Whitney U tests, as well as to compare fGCM concentrations of pregnant and non-pregnant females at NZP. All models were run with and without LP as a factor to determine any effects that location may have, and none were found.

3. Results

3.1. Biological validation of enzyme-immunoassays (EIA)

Although all tested EIAs showed a suitable increase in fGCM concentrations following the translocation event in both the female and male, the 11-oxoetiocholanolone II EIA displayed the biggest increases for both sexes when considered together (F: 274%, M: 81%) (Table 2). Both sexes had a peak increase in fGCM concentration in the first sample excreted post-

translocation (F: 3 days, M: 1 day). As such, all further analyses were conducted using this EIA.

Table 2. The median baseline and peak fGCM concentrations (µg/g WW) as well as the % increase for the five EIAs tested in the biological validation study. The peak fGCM concentration was found to be in the first sample deposited post-translocation in both the female (3 days post-translocation) and the male (1 day post-translocation).

	Animal 1 - Female			Animal 2 - Male		
	Base-line fGCM	Peak fGCM	% increase	Base-line fGCM	Peak fGCM	% increase
Cortisol	0.02	0.03	50%	0.03	0.13	351%
11-oxoetiocholanolone I	0.78	1.96	151%	0.79	0.64	−19%
11-oxoetiocholanolone II	1.37	5.11	274%	2.07	3.74	81%
5α-pregnane-3**β**,11**β**,21-triol-20-one	0.14	0.68	404%	0.29	0.39	36%
Corticosterone	0.18	0.28	52%	0.21	0.64	200%

3.2. Changes in fGCM concentrations post-defecation

Median fGCM concentrations fluctuated over time with maximum increases of 32.0% and 30.8% at six and 144 h compared to fGCM concentrations immediately following defecation. However, respective fGCM concentrations did not differ significantly over time ($F_{6,14} = 0.54$, $P = 0.77$) (Fig. 4).

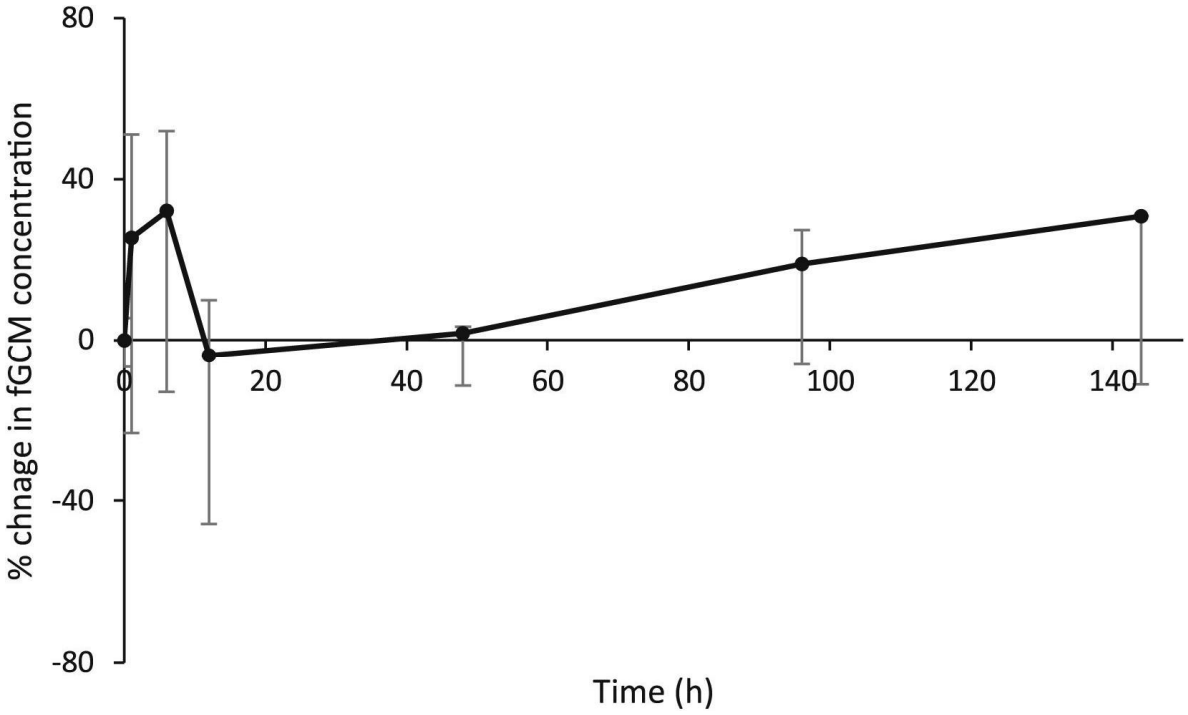

Fig. 4. Relative change (%) in fGCM concentration in tiger (*Panthera tigris*) faeces over time (0 h, 1 h, 6 h, 12 h, 48 h, 96 h, 144 h) post-defecation. The median, 25th, and 75th percentile values from each time point are shown and 100% concentration measured at time 0 was used to calculate the percent changes at all other time points.

3.3. fGCM concentrations of tigers at different locations

Tiger fGCM concentrations differed significantly between study locations (H_7 = 115.27, P < 0.001), with fGCM concentrations of animals at LP being significantly higher compared to all other sites (H_7 = 115.27, P < 0.001). Overall median fGCM concentration of LP animals (16.70 µg/g DW) were up to 22-fold higher than respective values from animals at the other seven locations (0.76 – 3.46 µg/g DW) (Fig. 5). With the exception of LP, there was no significant difference in fGCM concentrations between the South Africa and Indian study sites ($H = 52_{52}$, P = 0.474).

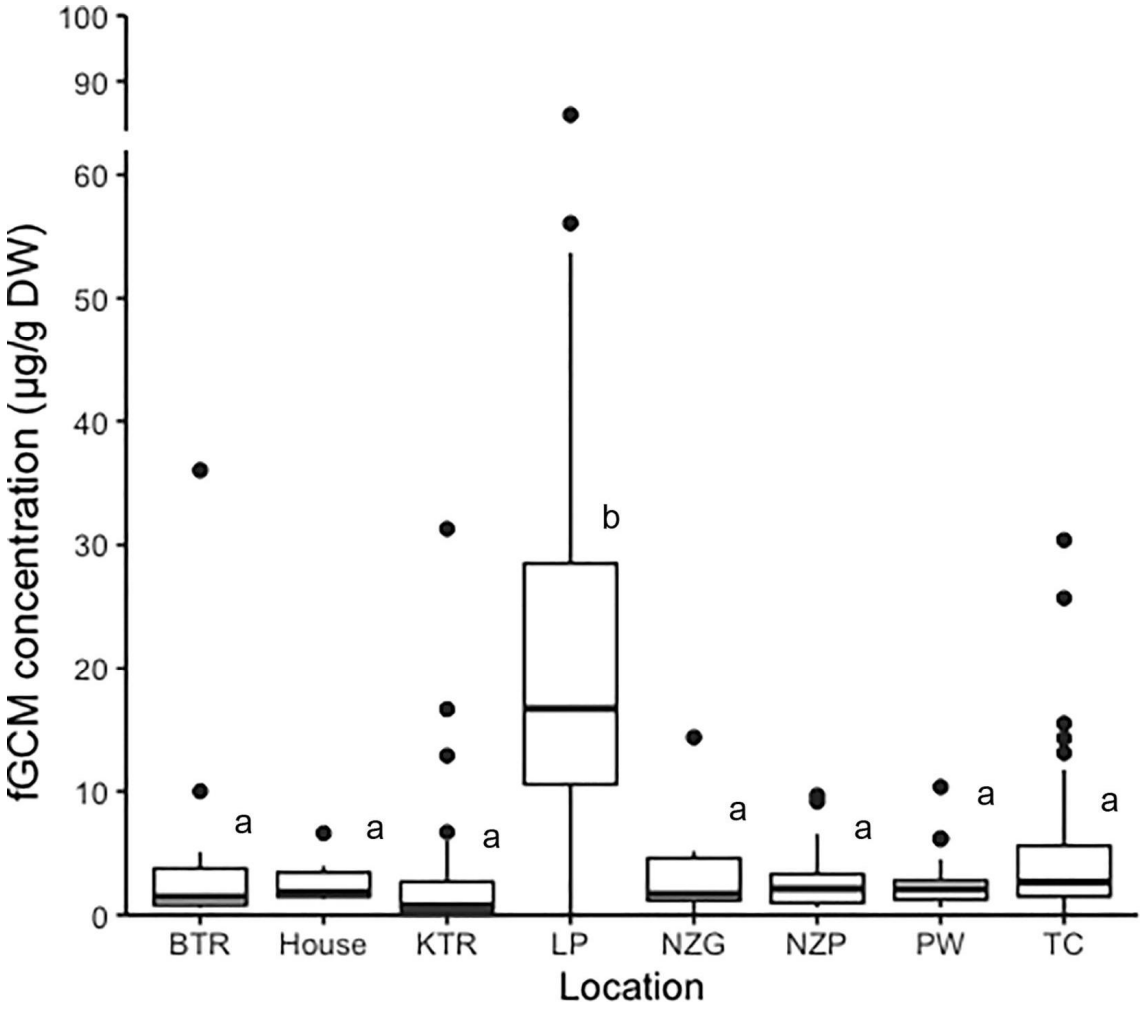

Fig. 5. Boxplots of grouped fGCM concentrations (µg/g DW) in tigers (*Panthera tigris*) at the eight different study sites. The boxes show the median value and the upper and lower quartile values; the whiskers show the 10th and 90th percentiles of the values, the dots outliers. Different superscripts indicate significant differences between sites. Sample numbers (N) = 20 (BTR), six (House), 36 (KTR), 65 (LP), eight (NZG), 39 (NZP), 38 (PW), 61 (TC).

3.4. fGCM concentrations of tigers on/off display

At LP the two on-display animals (26.65 ± 15.34 µg/g DW) showed significantly higher fGCM concentrations (W = 713, P < 0.01) compared to the two animals off-display (17.07 ± 14.85 µg/g DW). When comparing individual fGCM concentrations the two individuals on display (Kimberley: 27.78 ± 14.65 µg/g DW; Ashan: 25.61 ± 16.48 µg/g DW)

had significantly higher fGCM concentrations than one of the two off-display animals (Aaron: 12.17 ± 6.66 µg/g DW; H_2 = 12.008, P < 0.01), but not the other (Kiska: 21.98 ± 18.92 µg/g DW, W = 313, P = 0.155).

3.5. fGCM concentrations in relation to season, sex, and reproductive state

There was no significant difference in fGCM concentrations between seasons across the five South African study sites (winter: 11.21 ± 14.51 µg/g DW; summer: 8.31 ± 10.18 µg/g DW) (GLM, t_{177} = 0.218, P = 0.828). The Indian locations could not be included in the seasonal analysis as samples were only collected during one season.

There were no significant intra-site differences in fGCM concentrations between males (10.12 ± 11.73 µg/g DW) and females (15.92 ± 17.09 µg/g DW) (LP: W = 404, P = 0.105; PW: W = 139.5, P = 0.914; NZG: W = 6, P = 1.00; NZP: W = 55, P = 0.845; KTR: W = 105, P = 0.855; BTR: W = 52, P = 0.545) (Fig. 6). For seven of the eight sites, females showed larger variations in their fGCM concentrations compared to males.

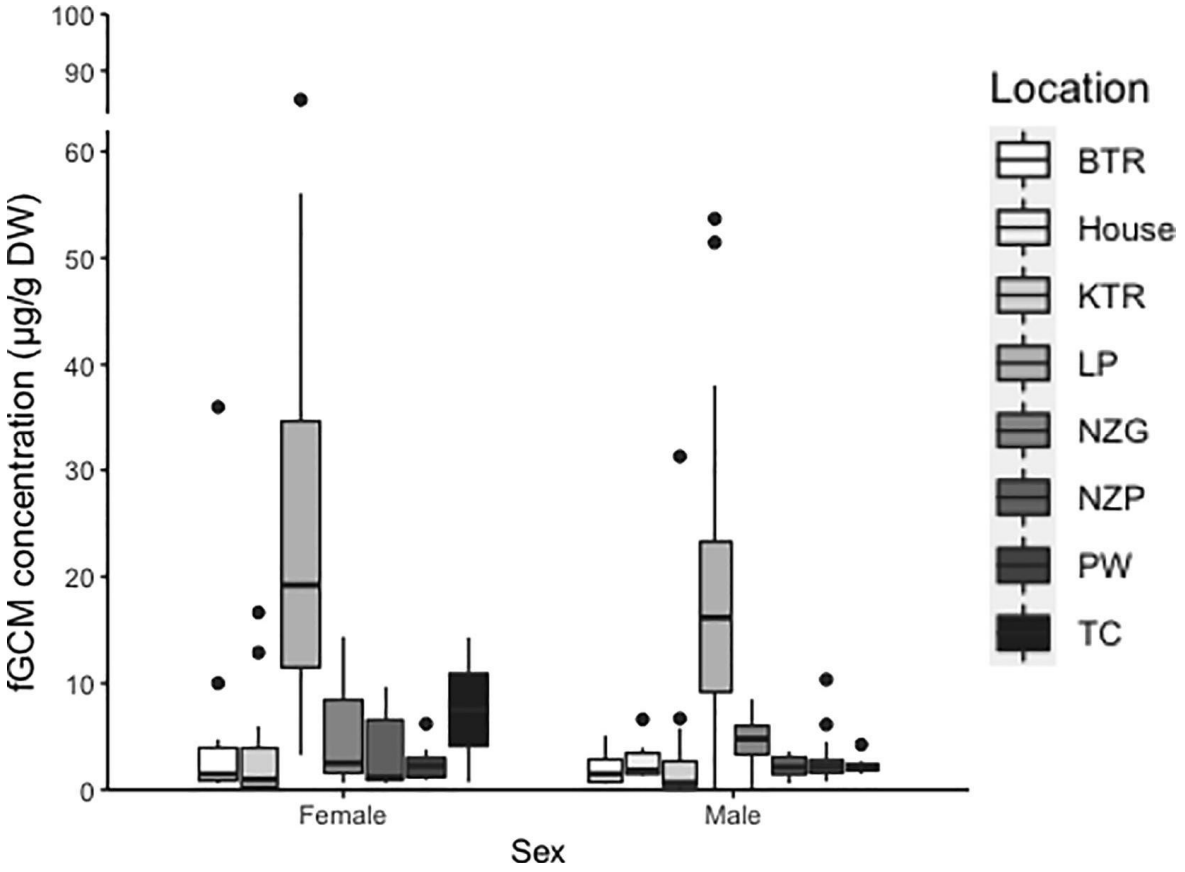

Fig. 6. Boxplot of fGCM concentration (µg/g DW) of male and female tigers (*Panthera tigris*) across sites. The boxes show the median value and the upper and lower quartile values; the whiskers show the 10th and 90th percentiles of the values. House is only represented on the male side as the single tiger there was male.

Pregnant females at NZP had significantly higher fGCM concentrations (H_2 = 17.89, P < 0.001) than their non-pregnant counterparts (pregnant: 12.07 ± 8.09 µg/g DW, non-pregnant: 3.90 ± 3.77 µg/g DW, W = 24, P < 0.01) as well as males (2.13 ± 1.01 µg/g DW, W = 19, P < 0.001) (Fig. 7).

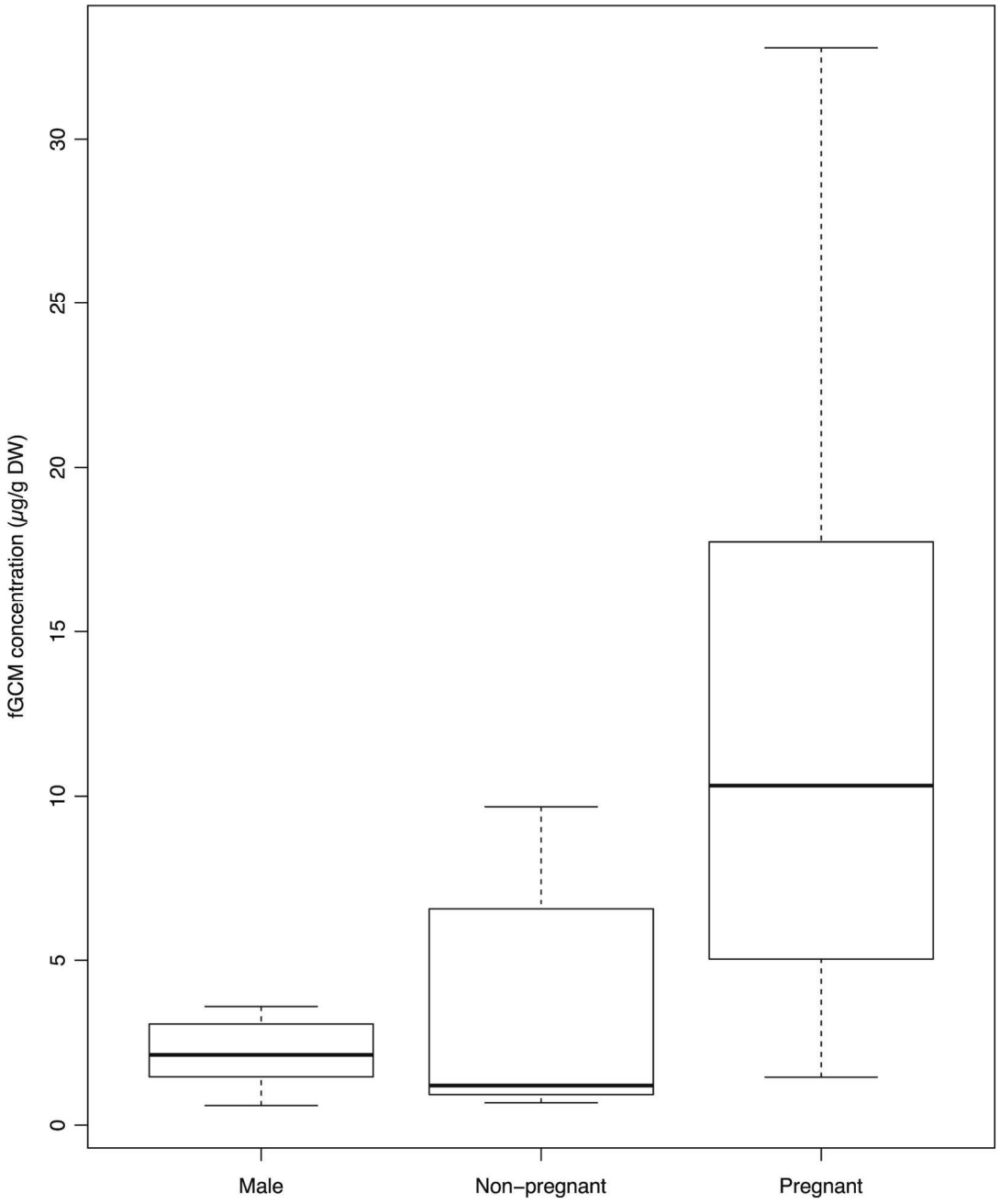

Fig. 7. Boxplot (median, 25th percentile, 75th percentile) of fGCM concentration (µg/g DW) of males, pregnant, and non-pregnant female tigers (*Panthera tigris*) at NZP. Different superscripts indicate significant differences between groups. N = 13 (Male), 9 (Non-pregnant), 18 (Pregnant).

4. Discussion

This study on tigers was the first to implement non-invasive fGCM monitoring in captive, re-wilded, and free-ranging animals in native (India) and exotic environments (South Africa). We successfully established an EIA for measuring fGCM concentrations in faecal samples of tigers and showed that fGCM concentrations can be reliably determined up to 6 days post-defecation. Furthermore, a comparison of fGCM concentrations in captive, re-wilded, and free-ranging tigers revealed that in and of itself, being in an exotic environment does not increase fGCM concentrations. However, this is site and population specific, with elevated

fGCMs found at one location. Additionally, sex and season do not influence fGCM concentrations, but reproductive status of females does.

This study used a biological validation, transportation in this case, to determine the most appropriate EIA for monitoring fGCM concentrations in tigers. This validation was successful for the 11-oxoetiocholanolone II assay detecting fGCMs with a 5β-3α-ol-11-one structure, which has also been used to measure fGCM concentrations in a variety of species including male leopards (*Panthera pardus*), (Webster et al., 2018), Samango monkeys (*Cercopithecus albogularis*) (Scheun et al., 2020), and giraffes (*Giraffa camelopardalis*) (Bashaw et al., 2016).

In contrast to previous studies investigating alterations in immunoreactive fGCM concentrations post-defecation in predatory species indicating either a sudden increase (African clawless otter, *Aonyx capensis;* Majelantle et al., 2020; African wild dogs, *Lycon pictus*; Crossey et al., 2018) or decrease (brown hyena, *Hyaena brunnea*; Hulsman, 2010; banded mongoose, *Mungos mungo*; Laver et al., 2012) within 24 h of the defecation event, fGCM concentrations within tigers remained constant up to 144 h post-defecation. This is consistent with similar studies on other members of the *Panthera* genus: both jaguars (*Panthera onca*) and leopards have been shown to have stable fGCM concentrations for up to a week post-defecation (Mesa Cruz, 2014, Webster et al., 2018). Our finding is in direct contradiction to Parnell et al. (2015) who found that fGCM concentrations in their study on tigers differed significantly after 48 h. However, Parnell et al. (2015) used a different EIA, and antibody specificities can contribute to overall different patterns in fGCM concentrations, as seen in Lexen (2008), thus making the results incomparable. Although the prolonged comparability of fGCM concentrations found in this study allows for a more practical solution to sample collection as transects can be walked once a week rather than every day with no concern for alterations in fGCM concentrations (Webster et al., 2018), the degradation results of this study were performed under constant environmental conditions (~17°C, no rainfall). Thus, further research into the rate of fGCM alteration in faeces exposed to varying environmental conditions in both winter and summer months should be conducted, as fluctuations in temperature, radiation, and UV exposure can all impact the rate of enzymatic bacterial metabolism of hormone metabolites (Lexen et al., 2008, Palme et al., 2013).

Due to the tigers at TC having to hunt for themselves and being more exposed to the environment and therefore experiencing more physical demands, we expected to see a difference in fGCM concentrations between the re-wilded and captive tigers. The lack of significance found in this study aligns with Sajjad et al. (2011), who found no significant difference in plasma cortisol concentrations between zoo tigers and tigers kept in a semi-natural environment where they had ample space but were still fed. While sample sizes were low and more should be collected in future studies, the lack of significance between TC and most of the other study sites may be due to the fact that all of these tigers were born in their respective settings and did not have to adjust to a new environment. It has been shown that in some species, genetic adaption to captive conditions can happen in a single generation, leading to behavioural and physiological adjustments that allow for better fitness (Håkansson and Jensen, 2005, Christie et al., 2012). To get an inclusive picture of the animal's welfare however, additional monitoring techniques to assess the health of captive

and free-ranging animals should be implemented, including measuring heart rate and behavioural observations (Keeling and Jensen, 2002), especially stereotyped behaviours (Pitsko, 2003, Clubb and Vickery, 2006). Like almost all reserves, zoos, and parks, TC has anthropogenic stressors from fences and maintenance work being performed. However, the stress from close human interactions was minimal, having low visitor levels year-round. Thus, places like TC offer opportunities for a novel approach of conserving tigers. It allows the animals to live a more natural life in an exotic environment, while being free from many anthropogenic pressures they face in the wild including habitat infringement, poaching, and persecution. Furthermore, it can enhance people's education and understanding of these cats as they can observe them performing behaviours such as hunting which they would not be able to in captivity.

Similar fGCM concentrations observed in this study at the majority of study sites indicate that captivity and management protocols are not main drivers of adrenocortical activity. In fact, these aspects are much less likely to cause increased fGCM concentrations than natural stressors like pregnancy. This contradicts what Naidenko et al. (2011) found in Siberian tigers, with wild tigers having significantly higher fGCM concentrations than captive tigers, which the authors explained by the wild tigers having higher metabolic activity due to their increased activity including hunting and other factors that also influenced their metabolism. In the same study, they found the wild tigers had the highest fGCM concentrations in winter when ambient temperatures decreased (Naidenko et al., 2011). The extreme conditions faced by wild tigers in the study by Naidenko et al. (2011) may explain the reported significant difference between wild and captive individuals compared to the more pleasant conditions experienced by tigers of this study. However, our finding is positive for tigers under human care, indicating that they do not seem to be under long-term stress that could negatively affect their fitness and survival. Nonetheless, as mentioned prior, all aspects of the animal's physiology and behaviour should be monitored to get a more inclusive understanding of their well-being. Additionally, although Narayan et al. (2013) found there to be no difference in fGCM concentrations between captive subspecies, we recommend genetic studies be conducted in South Africa to determine the specific subspecies being sampled to allow for more exhaustive comparisons to tigers elsewhere in the world

An exception to the comparable fGCM values found in free-ranging and captive tigers in this study are the four animals at LP. Although LP tigers were born in captivity, other factors such as enclosure size and quality, as well as proximity to both people and other animals may be the reason for the comparatively higher fGCM concentrations found in those animals. Where LP differed from the other sites was the proximity to visitors; the walkway past the cages was minimum 1.5 m closer here than at any other site. Moreover, the LP tigers on display, were bordered on one side by two lions, and by two jaguars on the other side, both possible competitors. A similar situation was shown to occur in clouded leopards (*Neofelis nebulosa*) when they were situated near potential predators (Wielebnowski et al., 2002), indicating that felids may be disturbed by the presence of top predators. Future studies should incorporate factors such as enclosure size, the proximity of human visitors and the presence of other predators, such as lions, to their analysis, as all of these have been shown to activate the physiological stress response (De Rouck et al., 2005, Vick et al., 2012, Vaz et al., 2017). Such information might assist in determining why populations such as the LP tigers showed such elevated fGCM concentrations.

The lack of winter/summer variation in fGCM concentrations in our captive study animals reflects what other studies have found on captive Siberian tigers (Byers et al., 1990, Naidenko et al., 2011). This is in contradiction to a study by Ivanov et al. (2017), where captive Siberian tigers had increased fGCM concentrations during lower ambient temperatures and only gradually adapt to temperature changes. In captivity, the general lack of seasonal variation in fGCM concentrations can most likely be explained by the constant availability of resources such as food and housing, which might minimise the impact of changing environmental factors (temperature/rainfall). Furthermore, captive tigers have likely adapted to these conditions and the presence of humans. Although exposed to season variation in temperature and vegetation cover, a constant prey base likely allowed TC tigers to maintain stable fGCM concentrations.

Pregnancy has been shown to cause higher fGCM concentrations in a number of species including Canadian lynx (*Lynx canadensis*) (Fanson et al., 2012), baboons (*Papio ursinus*) (Weingrill et al., 2004), and red squirrels (*Sciurus vulgaris*) (Dantzer et al., 2010). This same result was mirrored in our study, with pregnant females from NZP showing significantly elevated fGCM concentrations between sexes. The lack of significant difference in fGCM concentrations between sexes (when only including non-breeding females) align with results from Vaz et al. (2017), but contradict Narayan et al., 2013, Parnell et al., 2014 who both found that females had comparatively higher fGCM concentrations than males. This phenomenon might be result of potential sexual differences in hormone metabolism and excretion, as found in domestic cats (*Felis silvestris catus*) and dogs (*Canis familiaris*) (Schatz and Palme, 2001), in combination with differences in the specificity of antibodies used to quantify fGCMs. The higher variation in fGCM concentrations found in females in this study could be due to the reproductive cycles of females, as cyclic expression of reproductive hormones during the different phases can impact fGCM concentrations (Palme et al., 2005, Kinoshita et al., 2011). The larger variations in the fGCM concentrations of the females are most likely, like Narayan et al., 2013, Parnell et al., 2014 speculated, indicative of different stages in their reproductive hormone cycle (Kudielka and Kirschbaum, 2005, Palme et al., 2005, Fanson et al., 2012) which can lead to varying metabolic demands (Goymann et al., 1999, Touma et al., 2003, Cavigelli, 1999).

5. Conclusion

This study confirmed that fGCM concentrations can be reliably quantified in tigers using an 11-oxoetiocholanolone II EIA detecting fGCMs with a 5β-3α-ol-11-one structure and that tiger faecal samples can potentially be utilized up to a week after defecation, enabling easier sample collection and reliable results. While captive animals seem to have habituated to their surroundings, visitors included, this pattern was not repeated at one captive site, meaning that the physiological response to the same factors (humans/management regime etc.) might differ between populations, or subspecies, and should be kept in mind.

This study adds further support to the importance of tiger conservation, and the need to use a multi-faceted approach to understand tigers' welfare and how best to manage and save the species. As current conservation methods have several shortcomings, especially when used in a less than preferred environment, it is imperative that new and existing techniques be included to ensure the robust measurements of animal welfare and population trends.

By examining how different stress-associated variables, both environmental and anthropogenic in origin, affect tiger welfare, enhanced management protocols can be developed to ensure successful species conservation.

CRediT authorship contribution statement

Emma M. Jepsen: Formal analysis, Investigation, Writing - original draft. Juan Scheun: Supervision, Writing - review & editing. Martin Dehnhard: Resources, Writing - review & editing. Vinod Kumar: Methodology, Resources, Writing - review & editing. Govindhaswamy Umapathy: Methodology, Resources, Writing - review & editing. André Ganswindt: Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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