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Source: Wildlife Biology, 2020(1)

Published By: Nordic Board for Wildlife Research

URL: https://doi.org/10.1111/wlb.00646

Wildlife Biology 2020: wlb.00646
doi: 10.2981/wlb.00646

Subject Editor: Luca Corlatti. Editor-in-Chief: Ilse Storch. Accepted 20 January 2020

Using faecal glucocorticoid metabolite analyses to elucidate stressors of African wild dogs *Lycaon pictus* from South Africa

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There are few stressful factors which have been investigated to affect adrenocortical function in the African wild dog (AWD) Lycaon pictus. Understanding what animals perceive as stressors is important for not only the implementation of management practices promoting general animal welfare in captivity, but also because a prolonged stress perception is known to disrupt reproduction, immune function and ultimately pose a threat to survival. In this regard, faecal glucocorticoid metabolite (fGCM) measurements are commonly used as a non-invasive approach to assess the impact of factors which may be acting as perceived stressors in wildlife. This study was aimed at determining if there are significant differences in AWD fGCM concentrations as a result of sex, presence of absence of an injury, age-class, body condition, dietary provisioning (through stable nitrogen (δ15N) and carbon (δ13C) isotope analysis), hierarchal status, and setting (i.e. captive or free-ranging). A total of 47 faecal samples were collected immediately post-defaecation from 39 AWDs across four free-ranging sites (Hluhluwe-iMfolozi Park, Madikwe Game Reserve, Kruger National Park and the Waterberg), two permanently captive sites (Johannesburg and Pretoria Zoo), and four temporary captive holding facilities (Hoedspruit Endangered Species Centre and Maremani, Tembe and Zimanga bomas) in South Africa. Captive AWDs had distinctively higher fGCM levels than their free-ranging counterparts, regardless of sex, body condition, hierarchal status, age-class or dietary provisioning. The present study is the first to assess physiological stress responses across permanently captive, temporarily captive and free-ranging AWDs within the South African managed metapopulation, while incorporating the use of stable isotope analysis to quantify differences in dietary isotopic profiles between these different settings. Ultimately this demonstrates the usefulness of fGCM analysis as a tool for assessing animal welfare in both captive and free-ranging AWDs, and underpins the importance of understanding factors perceived as stressors for the management of the species.
Keywords: animal welfare, body condition scoring, faecal glucocorticoid metabolite monitoring, managed metapopulation, stress, wildlife management.

African wild dogs *Lycaon pictus*, hereafter referred to as AWDs, were once distributed throughout most of sub-Saharan Africa (Skinner and Chimimba 2005), but have since disappeared from most of their historic range (IUCN 2016). The reasons for this decline are relatively well-understood, with direct anthropogenic persecution ranking alongside habitat fragmentation as the major causes (Creel and Creel 2002, Woodroffe and Sillero-Zubiri 2012). Currently, the AWD is the most endangered carnivore in South Africa (IUCN 2016) and, outside of the Kruger National Park (KNP), has mostly been managed as one metapopulation since 1998 (Mills et al. 1998, Davies-Mostert et al. 2015). This approach has been successful in increasing the number of AWDs in South Africa (Davies-Mostert et al. 2015), while maintaining the genetic diversity of the species, (Tensen et al. 2019), and works by periodically translocating animals between game reserves which host AWD packs in order to mimic natural dispersal events as closely as possible (Gusset et al. 2006, Potgieter et al. 2015). This management practice, however, requires AWDs to be kept in temporary holding facilities. In these facilities, individuals from different source populations are kept together until social bonds between animals are formed (the intricacies of which are outlined in Potgieter et al. 2015 and Marneweck et al. 2019). This increases the probability of the newly formed pack remaining together and ultimately reproducing within the first year post-release (Gusset et al. 2006, Marneweck et al. 2019).

It has been shown that female AWDs held permanently in captivity exhibit significantly higher stress levels than their free-ranging counterparts (van der Weyde et al. 2016). Generally, captivity is known to be perceived as a stressor across different species due to factors such as a lack of environmental enrichment (Wielebnowski 2002), anthropogenic and increased noise levels (Owen et al. 2004, Francis et al. 2009, Barber et al. 2010), as well as on-exhibit display (Terio et al. 2004). For South African captive AWDs in particular, the managed metapopulation approach presents a unique scenario. Primarily, this is because some individuals are born into captivity and are permanently housed under these conditions (for example in zoos). While others which are translocated as part of the managed metapopulation are only held temporarily in captivity (mostly in bomas in conservation areas) after having lived under free-ranging conditions. These circumstances add an extra degree of complexity in terms of isolating factors which may act as AWD perceived stressor complexes across captive sites (which are known to differ based on different husbandry techniques (van der Weyde et al. 2016, Crossey et al. 2018), as individuals with differing individual life histories may respond differently to the same perceived stressor (Wingfield et al. 1998)).

Being almost completely obligate cooperative breeders that exhibit complex intra pack dynamics and inter pack variation, such as male versus female sex biased dispersal in different reserves (Creel and Creel 2002, Skinner and Chimimba 2005), AWD packs kept in captivity for any period of time require careful management. This is of particular importance when one considers the aggressive interactions which naturally occur between AWDs when there is an unestablished and/or unstable dominance hierarchy, with Creel et al. (1997) demonstrating that dominant AWDs had significantly higher stress levels than subordinates in packs which experienced as many as three changes in the dominant pair within a single year. Overall, this can be exacerbated by the fact that captive individuals are unable to escape from unfavourable conditions or aggressive conspecifics (Creel et al. 1997, Goymann and Wingfield 2004, Creel 2005), where injuries sustained (for e.g. by aggressive interactions) may also drive higher stress levels for these animals, as seen in other mammals (Ganswindt et al. 2010, Wolf et al. 2018). In addition, AWDs usually disperse from their natal packs when they are between one and two years old (McNutt 1996, Davies-Mostert et al. 2015). Thus, the inability of individuals to leave their packs as a result of permanent captive housing poses an unnatural scenario for these AWDs. Temporarily captive AWDs undergoing artificial pack formation, however, have usually already dispersed from their natal packs prior to being translocated (Potgieter et al. 2015, Marneweck et al. 2019). When subsequently being placed into temporary captivity with another group of opposite sex dispersers, those AWDs may also perceive stress, although its origin would be clearly different to potential responses seen in permanently housed AWDs.

Moreover, captive AWDs are reliant on humans to feed them, and limited variation in diet quality or quantity (Ashworth et al. 2009) could be a driver for nutrition-related stress in these animals. Variation in captive diets is likely to be reflected for captive South African AWDs between those housed temporarily in the managed metapopulation, which are fed a diet consisting of their natural prey species (David Marneweck unpubl.), and permanently captive AWDs, which are fed a wider range of food sources. These can range from livestock to natural AWD prey items depending on what is available at the permanent captive facility (Crossey et al. unpubl.). In this regard, stable isotope analysis of faecal material can be helpful to assess possible short-term differences in diet between captive and free-ranging AWDs. This isdue to isotopic fractionation, whereby elemental isotopes (such as $^{14}N/^{15}N$ and $^{12}C/^{13}C$) behave differently across environmental and physiological processes due to differences in atomic mass (McKinney 1950), ultimately providing an opportunity to trace the flow of nutrients through food webs (Post 2002, Codron et al. 2007).

The analysis of faeces to determine glucocorticoid metabolite (fGCM) concentrations is also a widely accepted tool for monitoring stress-related hormone levels in wildlife (Ganswindt et al. 2012). Following a recognized threat to homeostasis (referred to as a stressor), the production of stress hormones (glucocorticoids (GCs) in particular) occurs via the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Matteri et al. 2000, Sapolsky 2002, Boonstra 2005, Palme 2019). In combination with the activation of the sympathetic nervous system supporting the production of catecholamines, this acts as a rapid mechanism facilitating hyperglycaemia (Sapolsky 2002, Butcher and Lord 2004). A short-term adaptation for survival is thus utilized when the animal is faced with an acute stressor, such as fear of an imminent attack (Sapolsky 2002, Boonstra 2005). Under conditions where the action of a stressor becomes chronic, however, the negative effects of a prolonged stress response become apparent (Boonstra 2005). Known effects are the suppression of reproduction (which is of particular importance for AWDs, as the ultimate success of the species is based on functional reproductive packs (Marneweck et al. 2019)), decreased immuno-competency (Matteri et al. 2000, Sapolsky 2002, Dhabhar 2009), as well as the atrophy of muscle tissues (Möstl and Palme 2002), resulting in a compromised body condition that can be visually assessed in mammals (Ganswindt et al 2010, Wolf et al. 2018) and impaired cognition and alterations in behaviour (McEwen 2004). This, ultimately leading to long-term damage for the animal(s) in question (Lupien et al. 2009).

Using faecal material to quantify GC metabolites is favourable as it facilitates safe and feedback-free sampling from both captive and free-ranging individuals and allows for a more cumulative hormone signal to be assessed (Hulsman et al. 2011, Ganswindt et al. 2012). The methods for a reliable monitoring of fGCM concentrations have already been established for AWDs (Vlamings 2011, Crossey et al. 2018). Sex-specific differences in the metabolism of GCs are suspected for AWDs (van der Weyde et al. 2016). This is something that always needs to be considered when utilizing fGCM analysis (Touma and Palme 2005, Palme 2019). Other factors to consider in this regard include age (as senescence is associated with a general decline in the functioning of organs maintaining homeostasis (Sutanto and de Kloet 1994)), and individual heterogeneity in the stress response, which may alter with various other biotic and abiotic factors (Corlatti 2018).

The present study is the first to examine physiological stress responses of South African AWDs across free-ranging, permanently captive, and the managed metapopulation. This is also the first time that a SIA analysis has been used to assess dietary carbon and nitrogen isotopic profiles for the species. It was hypothesized that captive AWDs would exhibit significantly higher fGCM concentrations than their free-ranging counterparts. More specifically, it was predicted that the magnitude of this effect would differ based on whether the AWDs were housed in permanent captivity or temporarily for metapopulation management purposes. The very likely effect of different settings on fGCM concentrations was then evaluated by assessing interactions with other predictors included in the study. Differences in fGCM concentrations based on possible sex-specific metabolism of GCs were predicted, as were differences in fGCM concentrations because of variations in dietary isotopic carbon and nitrogen ratios. Dominant and older individuals, as well as animals considered to be in a poor body condition, were hypothesised to have comparatively higher fGCM concentrations than their subordinate and younger counterparts, and those with apparently better body conditions. Higher fGCM concentrations in injured individuals as opposed to those without injuries were also expected.

Material and methods

The present study was undertaken with the approval of the University of Pretoria Animal Ethics Committee (AEC) (Ethics clearance number: EC015-18), as well as the National Zoological Gardens (NZG) (Pretoria) Research Ethics and Science Committee (RESC) (Ethics clearance number: P17/08). Samples obtained from AWDs within the managed metapopulation were obtained with permission from the Endangered Wildlife Trust (EWT) (data sharing agreement number: 270).

Study animals and sites

The study was conducted on 39 AWDs across 10 study sites within South Africa (Fig. 1) from April 2018 to January 2019. These included two AWD permanent holding facilities (namely, Johannesburg Zoo (JHB Zoo) and Pretoria Zoo (PTA Zoo)), four temporary holding facilities (namely Hoedspruit Endangered Species Centre (HESC), Maremani Boma (Maremani), Tembe Boma (Tembe) and Zimanga Boma (Zimanga)), and four sites in which the animals were able to roam freely (free-ranging) that included Hluhluwe-iMfolozi Park (HiP), Madikwe Game Reserve (Madikwe), Kruger National Park (KNP) and an AWD pack from the Waterberg Biosphere Reserve (Waterberg) (Fig. 1). Individuals and packs are continually monitored at each site, allowing for the opportunistic collection of faecal material for fGCM and stable isotope analysis, as well as the collection of accompanying data such as the sex, hierarchal status (dominant or subordinate) based on the breeding status of pack members (determined by observed mate guarding or available data indicating breeding activities of individuals for the previous year, Creel and Creel 2002, age class (yearling or adult) Creel and Creel 2002), and body condition score (BCS) for each individual. Body condition scores were assessed using a

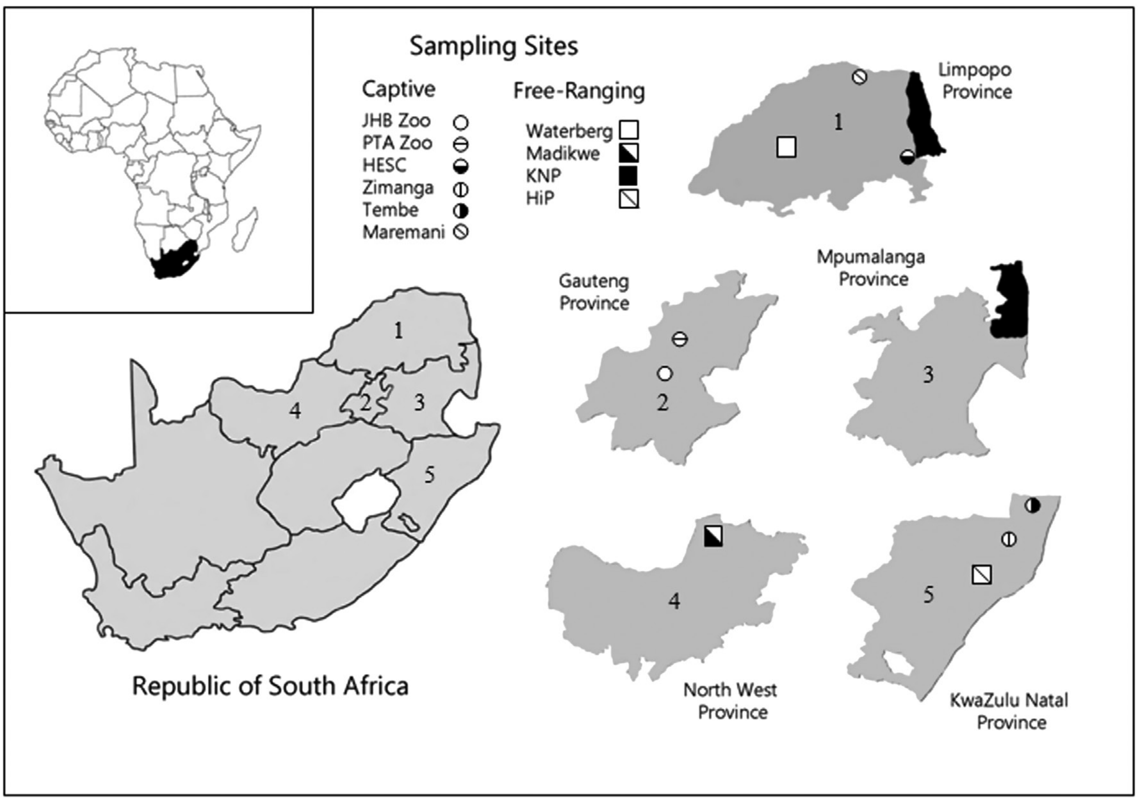

Figure 1. Sampling sites from which African wild dog (AWD) faecal samples were collected across South Africa.

set of visually determinable criteria adapted for determining the BCS of domestic dogs by the World Small Animal Veterinary Association (Freeman et al. 2011), with individuals being classified as either 'underweight', 'ideal' or 'overweight' in condition, based on an adaptation of the guidelines recommended by Laflamme (1997) and Mawby et al. (2001) (Supplementary material Appendix 1).

Faecal sampling

A total of 47 faecal samples were collected immediately post-defaecation from 35 known, apparently healthy AWD individuals directly after observing defaecation events (one to three samples per individual). An additional four samples were collected from four AWDs (one sample per individual) in the KNP with obvious physical injuries, two of which were caught in poachers' snares and had severe lacerations on their necks, and another two that had wounds from presumably fighting with other AWDs. No samples from assumed pregnant females were collected. Approximately 5 g of faecal material per sample was collected and frozen on site at −20°C within 24 h post-defaecation until further processing to prevent any alteration of faecal GC metabolites by suspected bacterial activity post-defaecation (Crossey et al. 2018). The number of samples collected from each site, as well as the sex of the individual from which the sample was collected are indicated in Table 1.

Steroid extraction and analysis

Frozen faeces were lyophilized, pulverized and sifted using a metal strainer in order to separate faecal powder from undigested material (Fiess et al. 1999). Between 0.050 and 0.055 g of faecal powder was then extracted with 3 ml of 80% ethanol in water by vortexing the suspension for 15 min. After centrifuging at 1500 g for 10 min, the resulting supernatant was transferred into sealed micro-centrifuge tubes for storage at −20°C until hormone analysis (Ganswindt et al. 2010). Steroid extracts were measured for fGCM concentrations using a competitive enzyme immunoassay for AWDs already established by ACTH challenge (Vlamings 2011), utilizing a cortisol-3-CMO:BSA antibody and a cortisol-3-CMO-DADOO-biotin label. Further details regarding assay components, cross-reactivities and assay characteristics are provided in Palme and Möstl (1997). Sensitivity of the assay was 1.2 ng g^{-1} dry weight (DW). Intra- and inter-assay coefficients of variation (CV), determined by repeated measurements of high and low quality controls was 5.67% and 6.90% (Intra-assay CV) and 12.40% and 12.81% (Inter-assay CV), respectively. Assay procedures were conducted in the Endocrine Research Laboratory, Univ. of Pretoria, South Africa, and followed published protocols (Ganswindt et al. 2002).

Stable isotope analysis sample preparation and analysis

Aliquots of faecal powder remaining from the steroid extraction procedure were weighed at ca 0.4–0.6 mg and placed in tin capsules which had been pre-cleaned in toluene. Samples were combusted at 1020°C in an elemental analyzer, coupled to a stable light isotope ratio mass spectrometer via a ConFlo IV system. Two laboratory running standards and a blank sample were run after every 11 unknown samples (Merck and Valine). These running standards were calibrated against international standards produced by International Atomic Energy Association (IAEA). All results are referenced to Vienna Pee-Dee Belemnite for carbon isotope values, and to air for nitrogen isotope values. Results are expressed in delta notation using a per mille scale using the following standard equation (Coplen 2011):

$$\delta X(\text{‰}) = \left[\left(R_{\text{sample}} / R_{\text{standard}}\right) - 1\right]$$

(where X = ^{15}N or ^{13}C and R represents $^{15}N/^{14}N$ or $^{13}C/^{12}C$, respectively).

Statistical analyses

Individual median fGCM concentrations were calculated if multiple samples were collected for an individual and, due to the small sample size, were subsequently used for all statistical analyses. An analysis of variance (ANOVA) and an ANOVA based on ranks were utilized a priori to determine if there were significant differences between AWD fGCM concentrations across free-ranging sites, as well as between permanently and temporary captive holding sites, respec-

Table 1. The sex, age-class, hierarchal status and number of African wild dog (AWD) faecal samples which were collected across four free-ranging and six captive sites in South Africa.

Setting	Site	Sex		Dominance status		Age class			
		n males	n females	n dominants	n subordinates	n yearlings	n adults	n AWDs	n samples
Free-ranging	KNP	8	4	1	11	0	12*	12	14
	HiP	1	4	0	5	2	3	5	5
	Waterberg	2	3	2	3	2	3	5	8
	Madikwe	0	1	0	1	1	0	1	1
	Total	11	12	3	20	5	18	23	28
Captive	JHB Zoo	2	2	2	2	1	3	4	4
	PTA Zoo	1	0	1	0	0	1	1	2
	HESC	1	0	0	1	0	1	1	3
	Maremani	2	0	0	2	2	0	2	2
	Tembe	0	3	1	2	2	1	3	3
	Zimanga	5	0	0	5	0	5	5	5
	Total	11	5	4	12	5	11	16	19

* These included four injured individuals (two with snare wounds and two with fight wounds).

tively. As no significant differences were found for fGCM concentrations between free-ranging sites ($F_{2,15} = 0.059$; $n = 19$; $p > 0.05$), these data were grouped as 'free-ranging'. Similarly, no significant differences in fGCM concentrations were found for AWDs being permanently or temporarily housed in captivity ($H = 0.260$; $df = 1$; $n = 16$; $p > 0.05$) and thus, these data sets were pooled into the category 'captive'. Median fGCM concentrations and residuals followed a normal distribution, and general linear models (GLMs) were, therefore, used to test the predictive potential of age-class (yearling versus adult), setting (captive versus free-ranging), sex (male versus female), BCS ('underweight', 'ideal' or 'overweight' condition), dietary $\delta^{15}N$ and $\delta^{13}C$ values (tested separately), and hierarchal status (dominant or subordinate) on the individual median fGCM concentrations of the 35 apparently healthy AWDs. Eight candidate regression models, including a global model including all factors and then individual models testing each factor separately, were tested against one another to assess their predictive potential on individual median fGCM concentrations (Table 2). Akaike information criterion (AIC) (Burnham and Anderson 1998) was used to identify the best model (Table 2). Due to the low sample size within each sub-group of captive versus free-ranging individuals, Student's t-tests were used in place of the inclusion of interactions in the selected GLM to test for differences in fGCM concentrations across all other predictors. Clustering of faecal dietary $\delta^{15}N$ and $\delta^{13}C$ values was determined visually. Possible statistical differences between $\delta^{15}N$ and $\delta^{13}C$ values between captive and free-ranging sites were assessed using a one-way ANOVA.

Statistical significance was set at alpha (α) = 0.05 and inferred at $p < 0.05$. The four obviously wounded AWDs were not considered for statistical analysis due to small sample size. Median fGCM concentrations for these individuals in relation to the cause of injury (poachers' snare versus fight wounds) were contrasted against fGCM values of the apparently healthy AWDs. Analyses were run using algorithms in R (<www.r-project.org>) with the use of the R Studio interface and the lm function in the package 'stats' ver. 3.3.1 as well as the ANOVA function in the package 'car' ver. 2.1.4 (Fox and Weisberg 2019). Illustrations were produced using Sigma Plot ver. 14.0 (Systat Software).

Table 2. Different general linear models (GLMs) testing the predictive potential of age-class, setting, sex, BCS, dietary $\delta^{15}N$ and $\delta^{13}C$ values, and hierarchal status on the individual median fGCM concentrations of African wild dogs (AWDs) in the present study which were compared using Akaike information criterion (AIC).

Candidate model	AIC	ΔAIC	Model weight	R-Squared
fGCM<-Setting	276.96	0.00	0.98	0.40
fGCM<-Sex + Age class + Setting + BCS + Hierarchy + Carbon + Nitrogen	284.87	7.91	0.02	0.36
fGCM<-Carbon	294.05	17.09	0.00	0.02
fGCM<-Nitrogen	294.64	17.68	0.00	0.01
fGCM<-BCS	295.15	18.19	0.00	0.01
fGCM<-Sex	295.57	18.61	0.00	0.01
fGCM<-Hierarchy	295.60	18.64	0.00	0.01
fGCM<-Age class	295.75	18.79	0.00	0.01

Results

The overall individual median fGCM concentration for the 39 AWDs monitored was 42.16 ng g^{-1} DW, with individual medians ranging from 17.59 to 153.43 ng g^{-1} DW (IQR = 23.97 ng g^{-1} DW). 'Setting' was the only significant predictor of differences in fGCM concentrations as determined across all of the GLMs tested, with the best selected GLM including only setting as a predictorfor differences in fGCM concentrations ($F_{1,33} = 23.50$; $n = 35$; $p < 0.05$, adj. $R^2 = 0.40$ (Table 2). The overall individual median fGCM concentration of captive AWDs (55.43 ng g^{-1} DW; range: 17.59–74.34 ng g^{-1} DW; IQR = 22.84) was 55% higher than the respective value for free-ranging AWDs (34.95 ng g^{-1} DW; range: 21.75–48.27 ng g^{-1} DW; IQR = 11.28 ng g^{-1} DW).

When grouped according to BCS, captive AWDs showed a higher overall individual median fGCM concentration compared to free-ranging animals in all three categories found ('underweight' condition: 139%, 'ideal' condition: 137% and 'overweight' condition: 138%), with significantly ($t_{21} = 4.28$; $n = 23$; $p < 0.05$) higher individual median fGCM levels for captive animals compared to free-ranging individuals scored 'ideal' condition (Fig. 2a). Both captive male and female AWDs had significantly higher (males: $t_{17} = 2.29$; $n = 19$; $p < 0.05$ and females: $t_{14} = 4.29$; $n = 16$; $p < 0.05$) overall individual median fGCM concentrations compared to their male and female free-ranging counterparts (the overall individual median for fGCM concentrations for captive males and females was 136% and 144%, respectively) (Fig. 2b). When considering age class, overall individual median fGCM concentrations were significantly higher ($t_{23} = 4.62$; $n = 25$; $p < 0.05$) in captive adults (136%) compared to free-ranging adults. Captive yearlings showed 144% higher overall individual median fGCM concentration compared to free-ranging yearlings (Fig. 2c), although this difference was not significant ($t_8 = -1.99$; $n = 10$; $p > 0.05$). In addition, subordinate individuals in captivity had significantly higher ($t_{25} = 3.89$; $n = 27$; $p < 0.05$) individual median fGCM concentrations with an overall increase of 38% compared to free-ranging subordinates (Fig. 2d). Although not statistically significant, dominant individuals in captivity exhibited an overall median fGCM concentration 45% higher than that of free-ranging dominant AWDs (Fig. 2d).

The obviously wounded AWDs which were excluded from the GLM showed fGCM concentrations ranging from 18.08–153.43 ng g^{-1} DW. The two individuals which were caught in poachers' snares had fGCM concentrations of 18.08 and 56.82 ng g^{-1} DW, respectively, whereas the AWDs with injuries arising from fights showed fGCM concentrations of 141.41 and 153.43 ng g^{-1} DW, respectively.

Faecal $\delta^{15}N$ and $\delta^{13}C$ values obtained for AWDs showed no distinct clustering (Fig. 3) as a result of diet for captive versus free-ranging individuals ($\delta^{15}N$ median: 9.89‰, range: 5.27–12.79‰; IQR = 1.71‰ and $\delta^{13}C$ median: −17.45‰, range: −23.03 to −13.80‰; IQR = 2.67‰). In addition, there were no significant differences in $\delta^{13}C$ median ($F_{1,33} = 0.45$; $n = 35$; $p > 0.05$) and $\delta^{15}N$ median ($F_{1,33} = 2.82$; $n = 35$; $p > 0.05$) values between captive and free-ranging AWDs.

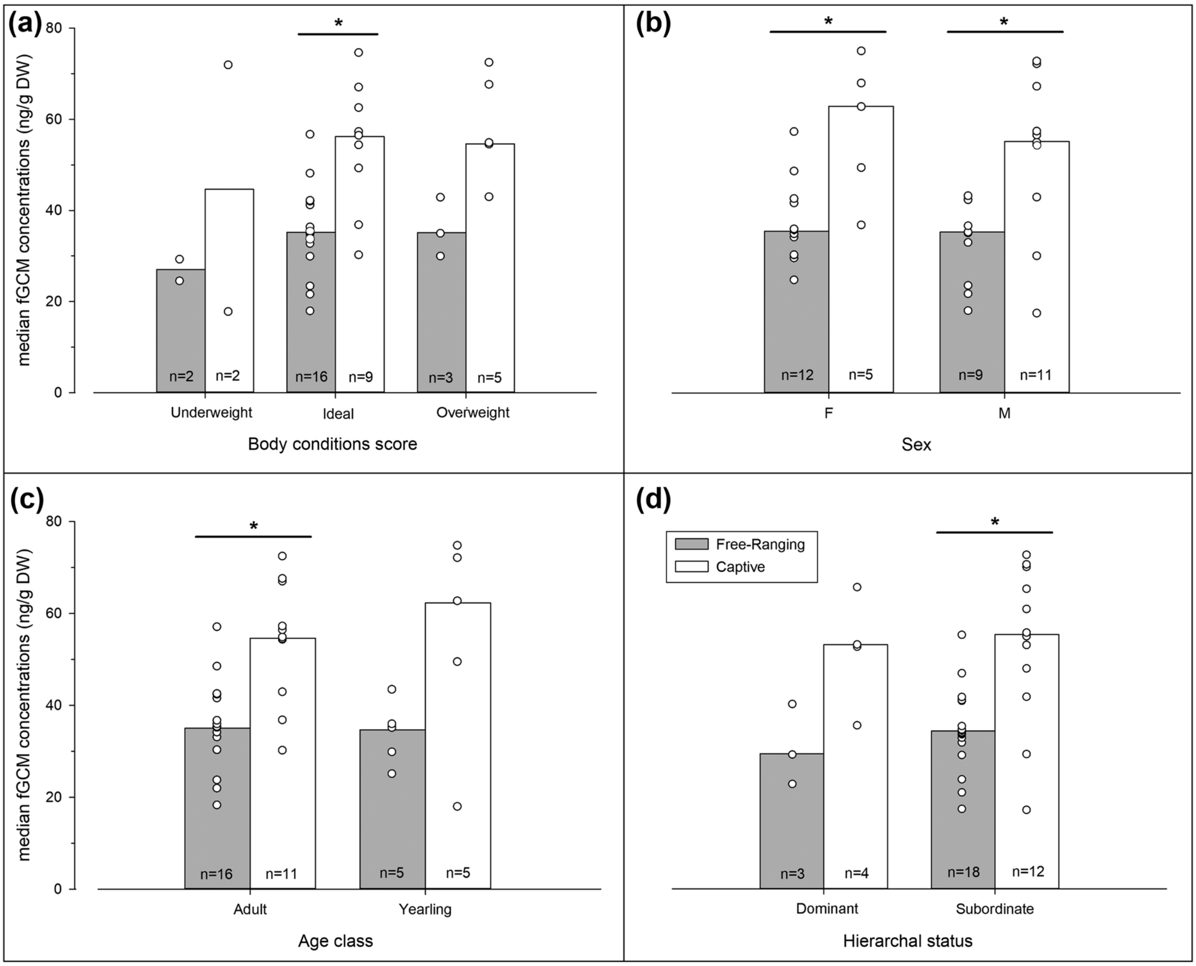

Figure 2. Dot-bar plot of individual median faecal glucocorticoid metabolite (fGCM) concentrations (ng g^{-1} DW) for African wild dogs (AWDs) categories per setting by body condition score (BCS) (a), sex (b), age class (c), and hierarchal status (d). Individual median fGCM concentrations are presented as points and the overall median fGCM concentrations for each category are represented as bars.

Discussion

Captive AWDs monitored in the present study had significantly higher fGCM concentrations than free-ranging individuals. Even when grouping the monitored animals based on BCS, sex, age-class, or hierarchal status, the only significant predictor driving differences in AWD fGCM levels was whether the setting for the animal was captive or free-ranging. Similar findings were obtained for female AWDs (van der Weyde et al. 2016), cheetahs *Acinonyx jubatus* (Terio et al. 2004) and spider monkeys *Ateles geoffroyi yucatanensis* (Rangel-Negrin et al. 2009).

Certain species cope with captivity better than others (Clubb and Mason 2003, Mason 2010, Morgan and Tromberg 2007). The present study shows that AWDs may be particularly prone to the negative effects associated with a chronic stress response in captive environments; regardless of whether being permanently kept or temporarily housed for reintroductions. The fact that the distinctively different settings for these two general types of housing still show no differences in AWD fGCM concentrations is, however, in line with the findings of van der Weyde et al. (2016) and Crossey et al. (2018), who demonstrated that differences in animal husbandry techniques underlie differences in the perceived stress responses of captive AWDs. Species such as the AWD that are naturally wide-ranging (Creel and Creel 2002) show the most evidence of stress in captivity based on the occurrence of stereotypic behaviours as a response to confinement in a sub-optimal environment (Clubb and Mason 2003, Mason and Latham

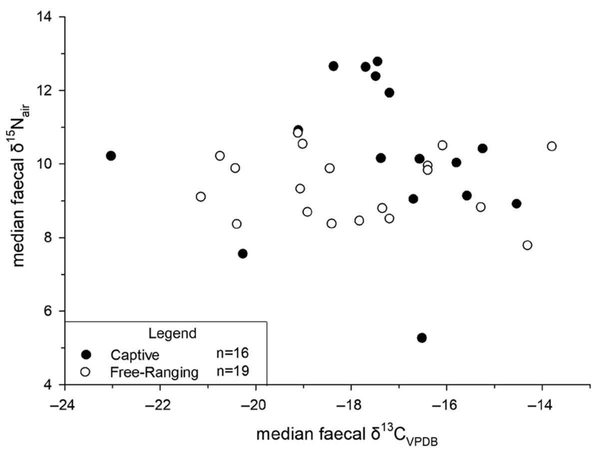

Figure 3. Median faecal $\delta^{15}N$ values plotted against median faecal $\delta^{13}C$ values obtained for each African wild dog (AWD) monitored.

2004, Swaisgood and Shepherdson 2005, Morgan and Tromberg 2007). The social structure exhibited by AWDs (Creel and Creel 2002) also means that the inability of permanently captive individuals to naturally disperse could destabilize pack social support and co-operation. Ultimately, this could result in a sufficiently large increase in conflict and competition that would be reflected in higher individual fGCM levels (Goymann and Wingfield 2004). Given this, a better understanding of what factors AWDs perceive as stressor complexes within captive environments, and how these can be mitigated, is vital to the welfare of these animals. From a management perspective this is of particular importance when considering the potential negative impact on AWD reproduction in a scenario where individuals are often in captivity for a defined period, and that are required to breed post-release. This possible effect has largely remained unconsidered by the managed metapopulation conservation strategy, but could have population-level consequences and thus should be noted in all decision-making moving forward.

There was no significant difference observed between the dietary $\delta^{15}N$ and $\delta^{13}C$ values between captive and free-ranging individuals, and any dietary-related small-scale differences observed were not identified for determining alterations in fGCM concentrations in these AWDs. The finding that permanently captive AWD diets showed no distinct clustering from free-ranging or temporarily captive AWDs may seem surprising as one would expect this to be clearly distinguishable. African wild dogs in captivity (regardless of the setting), however, appear indistinguishable from their free-ranging counterparts based on dietary $\delta^{15}N$ and $\delta^{13}C$ values, and it is therefore not surprising that this variable had no significant effect on fGCM concentrations. These results are also similar those of Kempster et al. (2007), who found no significant change in $\delta^{15}N$ and $\delta^{13}C$ values related with higher cortisol levels in food-restricted birds. The authors suggested that there may be a nutritional stress threshold below which changes in isotopic compositions are likely to be negligible, and this may be the case in the present study. Deschner et al. (2012), however, found that bonobo *Pan paniscus*, urinary GC levels increased as digestible energy intake (measured as $\delta^{13}C$ values) decreased. This shows how the broken down products of stored energy reserves (possibly resulting from the production of GCs, Palme et al. 2005, Palme 2019) can lead to observed changes in $\delta^{13}C$ values, and it is possible that this was not detected in the present study as only faecal material was analyzed.

African wild dog BCS was not significantly related to fGCM concentrations, which is surprising as body condition is described to significantly impact GC levels in other species such as horses, rodents and humans, with significantly higher GC levels found in overweight compared to underweight and healthy individuals (Fernandez-Real et al. 2002, Wang 2005, Anagnostis 2009, Hart et al. 2016). The fact that there was no significant difference in fGCM concentrations between the ideal and overweight AWDs monitored in the present study may be explained by the fact that being overweight may prove adaptive over the short-term for AWDs. For example, the presence of extra fat stores could act as a safeguard against the energetic costs associated with the high rates of kleptoparasitism by hyena *Crocuta crocuta* and lion *Panthera leo* experienced by free-ranging AWDs (Gorman et al. 1998). The sample size for underweight AWDs in the present study was limited to four individuals, making the potential effect of BCS on fGCM concentrations in these individuals difficult to assess. This possibly masked the difference that would be expected to be observed between overweight, ideal and underweight individuals as demonstrated in other previous studies. Future research should expand on this and determine whether or not this is the case over the long-term, particularly in permanently captive AWD packs.

Unlike Creel et al. (1997), the present study did not find any significant differences in fGCM concentrations between male and female AWDs. Creel et al. (1997) collected and analyzed 216 faecal samples from 22 females and 34 males over a two-year period in the Selous Game Reserve, Tanzania, and the more limited nature of the sampling conducted in the present study may be the reason that this difference was not detected. Van der Weyde et al. (2016) also showed that the fGCM levels in male and female AWDs are affected differently by periods of reproductive activity. The lack of sex-related differences in fGCM concentrations shown in this study might in fact be a result of insufficient sampling from individuals during various stages of reproductive activity. Such differences in fGCM concentrations between females and males can be expected during pregnancy, as this period appears energetically costly to females but not males (Creel and Creel 1991). The findings of the present study therefore suggest that differences in fGCM levels found between sexes in previous studies are rather a result of male and female AWDs showing differences in their responses to suspected stressor complexes, such as differences in the costs of reproduction (Creel et al. 1997, van der Weyde et al. 2016). This shows the importance of conducting sampling from known individuals and being aware of the possibility of sex-specific differences in fGCM metabolism which have been found in some species but not others (Palme 2019). Thus, making the inclusion of sex as a co-variable an important determinant in whether or not fGCM levels of both sexes should be pooled or analyzed separately in studies of this nature (Palme 2019).

The present study also found no significant effect of dominance status on fGCM concentrations in captive or free-ranging AWDs nor did age-class prove a significant predictor of stress levels in the AWDs monitored. The finding of no significant differences in fGCM concentrations between age classes may be an artefact of small sample size as a result of opportunistic sampling, however, this may also be due to a sampling bias whereby more young adults (possibly falling just outside of the 'yearling' category) were sampled. In such a case, any differences in age would not be distinct enough to determine if senescence is associated with a decline in the functioning of organs maintaining homeostasis for AWDs (Sutanto and de Kloet 1994). With respect to dominance status, the finding of no significant differences in fGCM concentrations between dominants and subordinates may again be due to an inadequate sample size, or the fact that the present study categorised individuals as dominant or subordinate based purely on their reproductive status. Although these results are in line with those of van der Weyde et al. (2016), who also found no relationship between social status and GC concentrations (even when controlling for breeding period), Creel et al. (1997) found significant differences between individuals of differing hierarchal status using a more dynamic approach over a distinctively longer period of time (Creel et al. 1997). These authors calculated dominance indices for pack members within each pack, observing as many as three changes in the alpha male within a pack in a single year. The observation that dominant AWDs seem to exhibit higher GC levels than subordinates year-round (Creel et al. 1997) has led to the suggestion that GCs are not responsible for reproductive suppression in the species (Creel 2005). Particularly as these heightened GC levels in dominant individuals are likely not entirely explained by aggressive interactions to maintain dominance, with such interactions generally confined to the mating period (Creel 2005). These results, however, were based predominantly on free-ranging AWD packs. Studies focusing on captive AWDs (de Villiers et al. 1997, van der Weyde 2013, van den Berghe et al. 2019), however, found no significant differences in fGCM concentrations between dominant and subordinate AWDs. Moreover, van den Berghe et al. (2019) found no significant difference in sperm quality of male AWDs based on hierarchal status, suggesting that reproductive suppression might be behaviorally mediated in AWDs. Marneweck et al. (2019), however, found that temporarily kept AWDs spending a significant amount of time in captivity after signs of social cohesion had been formed had ca 40% lower chance of reproduction in the first year post-release. This has led these authors to suggest that prolonged periods of captivity, coupled with being joined with unknown groups of AWDs, may elevate stress levels for these individuals for long enough to negatively impact reproduction in these AWDs. Further research would, however, be necessary in order to identify if there is a link between prolonged stress responses as a result of captivity (as suggested by the data in this study) and reproductive suppression in AWDs.

The four physically injured individuals monitored showed large scale differences in their measured stress levels as a result of what is considered an obvious stressor. Physical injuries are known to significantly increase stress levels in other African mammal species such as the African elephant *Loxodonta africana* (Ganswindt et al. 2010) and the giraffe *Giraffa camelopardalis* (Wolf et al. 2018). Of the injured AWDs monitored (all of which were in either 'ideal' or 'overweight' body condition), two were caught in poachers' snares, which, once removed, left deep-tissue lacerations. Surprisingly, neither of these two animals exhibited fGCM levels which fell outside the range of the 35 apparently healthy AWDs monitored. The other two AWDs which were removed from analyses, however, had injuries from fights with other AWDs. Both of these individuals exhibited fGCM levels around three-fold greater than the individual median fGCM concentration calculated for the apparently healthy AWDs. It therefore seems that not physical injury, per se, nor the apparent severity of the injury were responsible for the elevation of fGCM levels found in these animals, but rather the manner in which the injury was inflicted. While this supports the suggestion that social conflict and competition within a pack is likely to be reflected in higher fGCM levels, these results must be interpreted with caution due to the limited number of injured AWDs sampled.

Conclusion

The present study underpins the importance of elucidating stressors perceived by AWDs in order to ensure that especially captive individuals are carefully managed so that potential negative consequences associated with stress can be mitigated. In addition, the generated information should assist in focusing further on more long-term studies to assess endocrine responses to putative stressful circumstances in AWDs.

Acknowledgements – The authors would like to thank: 1) Nicole Hagenah-Schrader and Stefanie Ganswindt from the Endocrine Research Laboratory, University of Pretoria, Onderstepoort for technical assistance; 2) Carol Thobela, Lufuno Netshifhefhe, Tracy Rehse and Nathaniel Taweni (National Zoological Gardens, Pretoria), Agnes Maluleke and Kresen Pillay (Johannesburg Zoo); 3) Adine Roode, Linri Janse van Rensburg and Natasha Roscher (Hoedspruit Endangered Species Centre); 4) David Marneweck, Derek van der Merwe and Grant Beverley from the Endangered Wildlife Trust (EWT) and the Wild dog Advisory Group of South Africa, and 5) Dr. Louis van Schalkwyk, Dr. Peter Buss and Leanna Rossouw from SANParks for assistance with sampling logistics.

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