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In recent years, ecological studies have shown that oxidative status can have a significant impact on fitness components in free-ranging animals. This has raised awareness by conservation practitioners about the importance of identifying the factors associated with individual variation in markers of oxidative status because this might provide several potential benefits for conservation programmes. In this study, we measured five markers of oxidative status in the cheetah (*Acinonyx jubatus*), a carnivore species classified as vulnerable by the International Union for Conservation of Nature. We asked whether the five measures of oxidative damage and antioxidant blood-based markers are associated with a number of socioecological and environmental factors, including individual sex, age class, living condition (free-ranging vs. captive), restraint duration stress (i.e. capture duration stress), spatial tactic of males (territory holders vs. non-territory holders, i.e. floaters) and reproductive status of females (accompanied by offspring vs. solitary). Markers of oxidative damage were higher in those cheetahs that were physically restraint for a longer duration in the trap, indicating that oxidative stress may be increased by short-term unpredictable environmental stressors. Markers of oxidative damage were also higher in captive than free-ranging cheetahs, suggesting that oxidative stress might be a physiological mechanism underlying the detrimental effects of captivity on the health status of cheetahs. Variation of oxidative status markers was also significantly associated with individual age class, spatial tactic and reproductive status, opening new research avenues about the role of oxidative stress in influencing behavioural and life-history traits in cheetahs.

Key words: *Acinonyx jubatus*, ageing, captivity, cheetah, oxidative stress, restraint

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Knowledge of the stress resistance and health status of threatened and endangered species is fundamental for understanding and predicting the impact of ongoing environmental changes on population viability and for planning sustainable and successful conservation strategies. A promising and increasingly used approach of conservation practitioners is to identify physiological markers, i.e. measurable indicators of a given biological state, that reliably reflect individual

variation in the response to environmental stressors and fitness perspectives (Cooke *et al.*, 2013; Beaulieu and Costantini, 2014; Dantzer *et al.*, 2014).

Variation among individuals in marker values can arise from differences in genetic quality, conditions experienced during development, past or current health status and stressful experiences that may have caused cellular damage, increased secretion of stress hormones, and activation or suppression of the immune system (e.g. Hau *et al.* 2015; Marasco *et al.* 2017). It is increasingly advocated that physiological markers of allostatic load ('stress') may provide a valuable tool for long-term environmental monitoring of animal populations to assess the effects of environmental changes on individual health and to predict how individuals will cope with these ongoing changes (Romero, 2004; Busch and Hayward, 2009; Cooke *et al.*, 2013; Wingfield, 2013). Vertebrates exposed to unpredictable stressful situations increase their basal production of glucocorticoids (corticosterone or cortisol), whose action involves a high diversity of metabolic changes (e.g. Munck *et al.*, 1984; Romero *et al.*, 2009). When an individual is exposed to stressful conditions for a prolonged period, it may enter an emergency life-history stage in which resources are mostly used to sustain mechanisms activated to protect it from allostatic failure and to promote life-saving strategies essential to self-maintenance and survival (McEwen and Stellar, 1993; Wingfield *et al.*, 1998).

Quantification of glucocorticoid levels does not, however, provide an indication of the molecular damage that might be induced by excessive stress exposure or the activation or suppression of protective mechanisms against it. In recent years, there has been growing interest in understanding the roles of molecular oxidative damage (caused by pro-oxidant chemicals like free radicals) and antioxidant mechanisms as mediators of life-history trade-offs (reviewed in Costantini, 2008, 2014; Isaksson *et al.*, 2011; Speakman *et al.*, 2015). Many studies carried out on free-ranging animals demonstrated significant links between oxidative status markers and key life-history traits. For example, higher values of some blood oxidative damage markers were associated with high reproductive effort (Georgiev *et al.*, 2015), reduced survival and reproductive output (Vitikainen *et al.*, 2016) or reduced investment in eggs and offspring (Montoya *et al.*, 2016), while high values of plasma antioxidant capacity were associated with higher survival perspectives (Saino *et al.*, 2011). Concomitant to this research on the ecological relevance of oxidative stress, there has also been growing interest in the use of markers of oxidative status to identify threats to animal populations and to maximize the success of wildlife management (Beaulieu and Costantini, 2014). For example, higher oxidative damage was associated with reduced sperm quality (Helfenstein *et al.* 2010), *Plasmodium* infection (Isaksson *et al.* 2013), viral diseases (Sebastiano *et al.*, 2017) and human disturbance stemming from ecotourism (Semeniuk *et al.*, 2009; French *et al.*, 2017), while higher plasma antioxidant capacity was associated with coccidian infection (Pap *et al.*, 2011) and increasing population size (Beaulieu *et al.*, 2013).

This research has generally proven difficult because of the multitude of molecules involved in the regulation of oxidative status (Halliwell and Gutteridge, 2015). Another major limitation to understanding the biological relevance of oxidative status regulation in free-ranging animals is the paucity of studies that (i) measure both multiple markers of oxidative status and socioecological variables, which might shed light on the causes of within-group variation in physiological status and (ii) assess the extent to which markers change when an individual is being exposed to an unpredictable source of acute stress, such as physical restraint when free-ranging animals are captured for sampling or collaring.

The cheetah (*Acinonyx jubatus*) is a carnivore species that is classified as vulnerable by the International Union for Conservation of Nature (IUCN). However, due to the continuous population decline associated with a decrease of suitable habitats and an increase of conflicts with humans, it has recently been suggested that its IUCN status should be uplisted to endangered (Durant *et al.*, 2016). Here, we quantified five commonly used blood-based markers of oxidative status in free-ranging Namibian cheetahs and in captive cheetahs held in large enclosures in their natural environment on Namibian farms. The markers were (i) serum reactive oxygen metabolites, a marker of oxidative damage generated early in the oxidative cascade, (ii) serum protein carbonyls, a marker of oxidative damage to proteins, (iii) serum non-enzymatic antioxidant capacity to cope with hypochlorous acid (HOCl) *in vitro*, (iv) the activity in whole blood of the enzyme superoxide dismutase (sod), which prevents oxidation due to superoxide radical, and (v) the activity in whole blood of the enzyme glutathione peroxidase (gpx), which prevents oxidation due to hydrogen peroxide and organic hydroperoxides. All the chosen markers have been shown to be associated with survival estimates, life-history traits (e.g. reproductive effort, individual age), or exposure to abiotic stressors (e.g. thermal stress) in a number of vertebrates (reviewed in Costantini, 2014).

The first goal of this study was to determine whether our five measures of oxidative damage and antioxidant blood-based markers vary with individual sex, age class, living condition (free-ranging vs. captive) and restraint duration stress, i.e. capture duration stress. Second, we asked whether within-sex variation in markers is explained by the spatial tactic of males (territory holders vs. non-territory holders, i.e. floaters) and by the reproductive status of females (accompanied by offspring vs. solitary). All analyses were performed while controlling for a number of potential confounding variables that may affect markers of oxidative status, such as date and time of day of blood sampling, time elapsed from blood sampling to storage in the field and storage duration.

Blood samples of free-ranging cheetahs (53 individuals, 63 samples) were collected between 2012 and 2016 on commercial farmland in east-central Namibia (21°45′–22°45′S and 16°30′–18°30′E), whereas blood samples of 23 captive cheetahs were either collected in the same area or further north at the AfriCat Foundation, a non-profit conservation facility for carnivores. Samples of cheetahs used in the present study comprised 6 males and 5 females classified as sub-adults (age class 5; 13–23 months of age), 13 males and 5 females classified as young adults (age class 6; 24–42 months of age), 26 males and 14 females classified as prime adults (age class 7: >3.5–7.0 years of age) and 7 males and 10 females classified as old adults (age class 8: >7.0 years of age). The age of cheetahs was estimated as described in Caro (1994). Free-ranging cheetahs were trapped using box traps. These traps were equipped with an electronic device that sent the time via SMS when the gates of the trap closed. Once captured, cheetahs were kept in the box traps in the shade for several hours or overnight until the research team gathered to collect blood samples and to fit GPS collars to adult animals. This physical restraint was used to test whether markers of oxidative status are sensitive to restraint duration, which simulates individual exposure to an unpredictable stressor. Captive cheetahs were not restraint before immobilization but darted when they approached the fence to be fed, and used as comparison to free-ranging cheetahs. Free-ranging and captive animals were immobilized by remote intramuscular injection using a dart gun. The free-ranging animals were immobilized with a combination of 0.06 mg/kg medetomidine hydrochloride (Medetomidine 10 mg/ml, Kyron Laboratories, South Africa) and 3.2 mg/kg ketamine (Ketamine 1G, Kyron Laboratories, South Africa) and the captive animals were immobilized with 0.03 mg/kg medetomidine hydrochloride (Medetomidine 20 mg/ml, Kyron Laboratories, South Africa) in combination with 1.2 mg/kg zolazepam/teletamine (Zoletil, Virbac, South Africa). Blood samples were taken from free-ranging and captive cheetahs between 20 min and 35 min after darting, which is a timeframe during which there are not significant changes in oxidative status markers (Costantini, 2014). After approximately 45–60 min, the animals were given an antidote (free-ranging cheetahs 0.11 mg/kg and captive cheetahs 0.075 mg/kg atipamezole; Antisedan, Pfizer, South Africa) and observed until they had fully recovered from anaesthesia. Blood samples were taken both with non-heparinised and EDTA-Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and transported to the laboratory at the field station in a cooler box. At the field station laboratories, non-heparinised tubes were spun to separate serum from blood clots. Serum and whole EDTA blood samples were stored in liquid nitrogen in Namibia. Samples were transported to Germany in full compliance with the Convention on International Trade in Endangered Species (CITES) and stored at −80°C until laboratory analysis. All experimental procedures were approved by the Internal Ethics Committee of the Leibniz Institute for Zoo and Wildlife Research (IZW, Berlin, Germany) (permit number: 2002-04-01) and the Ministry of Environment and Tourism of Namibia (permit numbers: 1514/2011, 1689/2012, 1813/2013, 1914/2014 and 2067/2015).

The five oxidative damage and antioxidant blood-based markers were assessed using commercially available kits commonly applied to vertebrates (e.g. Costantini *et al.*, 2011; Saino *et al.*, 2011; Beaulieu *et al.*, 2013; Isaksson *et al.*, 2013; Vitikainen *et al.*, 2016) following the manufacturer's instructions unless otherwise mentioned. Reactive oxygen metabolites (mainly organic hydroperoxides) were measured in serum using the d-ROMs assay (Diacron International, Grosseto, Italy). Values were expressed as mM H_2O_2 equivalents and as mM H_2O_2 equivalents per mg of proteins to estimate reactive oxygen metabolites generated from oxidation of biomolecules of non-protein origin, such as fatty acids. Serum protein carbonyls were measured using the Protein Carbonyl Colorimetric assay (Cayman Chemical Company, Ann Arbor, MI, USA), which is based on the protocol of Levine *et al.* (1990). Values were expressed as nmol per mg of proteins. The OXY-Adsorbent test (Diacron International) was used to quantify the ability of non-enzymatic antioxidant compounds present in the serum to cope with the *in vitro* oxidant action of HOCl (an endogenously produced oxidant). Values were expressed as mM of HOCl neutralized and as mM of HOCl neutralised per mg of proteins to estimate the antioxidant potential of micromolecular antioxidants (e.g. vitamins, carotenoids, glutathione) without the contribution of proteins (i.e. non-enzymatic micro-molecular antioxidant capacity). The Ransod assay (RANDOX Laboratories, Crumlin, UK) was used to quantify the activity of superoxide dismutase (sod) in haemolysate obtained from whole blood. The activity of sod was expressed as units per mg of proteins. The Ransel assay (RANDOX Laboratories, Crumlin, UK) was used to quantify the concentration of gpx in haemolysate obtained from whole blood. This assay is based on the original method of Paglia and Valentine (1967) and analyses were carried out according to previous studies (e.g. Costantini *et al.*, 2011). The kinetic reaction was followed for 3 min by reading at 340 nm. A blank reaction was subtracted from the sample absorbance. Values were expressed as units of gpx per mg of proteins. The Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with albumin as a reference standard was used to quantify the concentration of proteins in either sera or haemolysates. Quality controls were included in all assays performed.

All statistical analyses were performed using R (R Core Team, 2013). General linear mixed models (lmer function in R) were

used to assess relationships between each oxidative status marker and the following predictor variables: sex, age class, living condition (free-ranging vs. captive), sampling date, time of day of blood sampling, restraint duration (minutes elapsed from capture to blood sampling), minutes elapsed from blood sampling to storage and storage duration. The factor individual was included as a random factor because from one individual we had three repeated measurements and from three individuals we had two repeated measurements. We also had three more individuals with two repeated measurements, but these were not included in the mixed models because of missing information on other variables such as restraint duration. These data were, however, considered in the analysis of within-individual changes over time. Adult males were categorized according to their spatial tactic, i.e. whether they were territory holders or floaters, and the *t*-test was used to compare markers of oxidative status between them. The *t*-test was also used to compare markers of oxidative status between lactating and non-lactating females accompanied by offspring and being alone. Females are lactating their offspring up to 4 months of age, after that the offspring feed alone on meat. Given the moderate sample size for each category, interactions between main factors were not included in the models.

Both markers of oxidative damage (reactive oxygen metabolites and protein carbonyls) and the serum non-enzymatic antioxidant capacity increased with time of physical restraint duration, while both antioxidant enzymes (sod and gpx) were not related to restraint duration (Table 1 and Fig. 1).

Older cheetahs had lower concentration of reactive oxygen metabolites (standardized by protein concentration), protein carbonyls and of non-enzymatic micro-molecular antioxidant capacity of serum, whereas they had higher activity of sod (Table 1 and Fig. 2). The number of cheetahs of which we had repeated measurements was low for statistical analyses. The average changes (value of a given marker recorded when the individual was older minus that recorded when the same individual was younger) of the four markers that were significantly associated with age class were as follows: −0.02 (SD ± 0.04) for reactive oxygen metabolites (n = 5), −0.68 (SD ± 1.60) for protein carbonyls (n = 5), −0.76 (SD ± 3.54) for the non-enzymatic micro-molecular antioxidant capacity (n = 5) and +0.16 (SD ± 0.45) for sod (n = 7). The time elapsed between the collections of the two blood samples ranged from 265 days to 1387 days.

Captive cheetahs had higher concentration of reactive oxygen metabolites standardised by protein concentration and of protein carbonyls than free-ranging cheetahs (Table 1 and Fig. 3). Males had lower non-enzymatic micro-molecular antioxidant capacity of serum and higher activity of gpx than females (Table 1). Finally, gpx activity was higher in samples stored for a longer duration (Table 1).

Territory holders had significantly higher protein carbonyls (t = 2.159, P = 0.044) and lower sod activity (t = −2.487, P = 0.018) than floaters (Fig. 4). Similar results were obtained when covariates that affected significantly protein carbonyls (restraint duration and age, see Table 1) and sod (age, see Table 1) were included in the models (results not shown). The non-enzymatic micro-molecular antioxidant capacity was significantly lower in floaters only when age class and restraint duration were included in the model as covariates (t = 2.208, P = 0.036, Fig. 4). Territory holders and floaters did not differ for all others markers irrespective of whether covariates were included or not in the models (all P-values ≥ 0.10).

Blood-based markers of oxidative status did not differ between free-ranging females accompanied by their offspring and solitary females (all P-values ≥ 0.09). There was only a marginal significant difference in the serum non-enzymatic antioxidant capacity, with females accompanied by offspring having higher values than solitary females (t = 2.193, P = 0.05). Within the group of females accompanied by offspring, those that were lactating had generally lower oxidative damage, non-enzymatic antioxidant capacity and gpx activity than post-lactating females (Table 2). The low sample sizes prevented us to run statistical comparisons, but effect size estimates represented by r Pearson show that according to Cohen (1988), differences between lactating and post-lactation females were large (Table 2).

This is the first study that has explored the socioecological and environmental predictors of multiple markers of oxidative status in a large free-ranging carnivore species. Restraint in a confined space, such as a cage or a box trap, is perceived as a stressful condition by animals (Glavin *et al.*, 1994; Wingfield *et al.*, 1998). Capture and restraint stress, i.e. acute stressors, have become widely used by ecophysiologists to examine how vertebrates respond to unpredictable environmental stressful challenges (i.e. labile perturbation factors; Wingfield *et al.*, 1998). We found that prolonged restraint resulted in higher values of oxidative damage markers, suggesting that one outcome of exposure to a short-term unpredictable stressor in adult cheetahs is an increase in blood oxidative stress. This result is in line with experimental restraint studies of laboratory rats, which demonstrated increased oxidative damage in plasma after a single restraint of 6 h (Zaidi *et al.*, 2003). The higher non-enzymatic antioxidant capacity in cheetahs restrained for a longer duration might indicate a mobilization of non-enzymatic antioxidants among tissues, possibly to buffer the lack of response of antioxidant enzymes, since neither sod nor gpx had any association with restraint duration. The activity of sod and gpx is controlled at both transcriptional and post-transcriptional levels (Halliwell and Gutteridge, 2015). Thus, the duration of the restraint might have been too short to detect a response at these levels. It might also be that cheetahs did

Table 1: Outcomes of linear mixed models used to detect the significant predictors of blood oxidative status markers. Significant *P*-values are shown in bold; marginally significant results are shown in italics

ROMs	Sex	Female	Male	0.070	0.054	1.299	0.199
mM H_2O_2 equivalents	Age class	5	6	−0.115	0.088	−1.306	0.197
			7	−0.060	0.078	−0.769	0.445
			8	−0.026	0.104	−0.246	0.807
		6	7	0.055	0.069	0.795	0.430
			8	0.089	0.096	0.929	0.357
		7	8	0.035	0.074	0.471	0.639
	Living condition	Captivity	Free-ranging	−0.134	0.105	−1.269	0.209
	Sampling date			0.0002	0.0004	0.373	0.710
	Storage duration			−0.00009	0.00007	−1.420	0.161
	Sampling time			−0.00009	0.00012	−0.829	0.411
	Time from sampling to storage			−0.000008	0.000061	−0.131	0.896
	Restraint duration			0.00011	0.00004	2.562	**0.013**
ROMs	Sex	Female	Male	−0.00085	0.00980	−0.087	0.931
mM H_2O_2 equivalents/mg proteins	Age class	5	6	−0.042	0.015	−2.793	**0.007**
			7	−0.030	0.014	−2.163	**0.035**
			8	−0.039	0.018	−2.161	**0.035**
		6	7	0.013	0.012	1.044	0.301
			8	0.004	0.017	0.206	0.837
		7	8	−0.009	0.013	−0.745	0.459
	Living condition	Captivity	Free-ranging	−0.047	0.018	−2.615	**0.012**
	Sampling date			−0.00003	0.00006	−0.506	0.622
	Storage duration			−0.000007	0.0000103	−0.677	0.502
	Sampling time			0.00001	0.00002	0.573	0.572
	Time from sampling to storage			−0.000008	0.000011	−0.805	0.424
	Restraint duration			0.000029	0.000006	4.904	**<0.001**
Protein carbonyls	Sex	Female	Male	0.293	0.315	0.932	0.356
nmol/mg proteins	Age class	5	6	−1.462	0.496	−2.948	**0.005**
			7	−1.173	0.447	−2.625	**0.011**
			8	−2.487	0.587	−4.235	**<0.001**
		6	7	0.288	0.399	0.723	0.473
			8	−1.03	0.546	−1.878	*0.066*
		7	8	−1.31	0.41	−3.200	**0.002**
	Living condition	Captivity	Free-ranging	−1.350	0.604	−2.234	**0.030**
	Sampling date			−0.0015	0.0022	−0.654	0.517

(Continued)

Table 1: continued

	Storage duration			0.00021	0.00035	0.614	0.541
	Sampling time			−0.0003	0.0007	−0.468	0.642
	Time from sampling to storage			−0.000009	0.000342	−0.026	0.979
	Restraint duration			0.00054	0.00022	2.428	**0.020**
OXY	Sex	Female	Male	−20.47	12.16	−1.684	0.099
mM HOCl neutralised	Age class	5	6	−1.33	19.29	−0.069	0.945
			7	−13.85	17.35	−0.798	0.428
			8	−10.82	22.83	−0.474	0.637
		6	7	−12.52	15.43	−0.811	0.421
			8	−9.49	2.12	−0.448	0.656
		7	8	3.03	15.98	0.189	0.851
	Living condition	Captivity	Free-ranging	14.75	23.44	0.629	0.532
	Sampling date			0.055	0.088	0.633	0.530
	Storage duration			−0.023	0.014	−1.664	0.101
	Sampling time			−0.034	0.026	−1.309	0.196
	Time from sampling to storage			−0.0008	0.0133	−0.064	0.949
	Restraint duration			0.018	0.009	2.022	**0.048**
OXY	Sex	Female	Male	−1.175	0.457	−2.575	**0.013**
mM HOCl neutralised/mg proteins	Age class	5	6	−1.565	0.746	−2.097	**0.040**
			7	−1.775	0.663	−2.679	**0.010**
			8	−2.406	0.882	−2.729	**0.008**
		6	7	−0.210	0.586	−0.359	0.721
			8	−0.841	0.816	−1.030	0.307
		7	8	−0.631	0.621	−1.015	0.314
	Living condition	Captivity	Free-ranging	−1.268	0.884	−1.434	0.157
	Sampling date			−0.002	0.003	−0.668	0.506
	Storage duration			0.000004	0.000532	0.008	0.994
	Sampling time			−0.00005	0.00101	−0.052	0.959
	Time from sampling to storage			−0.0005	0.0005	−1.010	0.316
	Restraint duration			0.0012	0.0004	3.567	**<0.001**
SOD	Sex	Female	Male	0.014	0.013	1.035	0.305
Units/mg proteins	Age class	5	6	0.033	0.021	1.550	0.126
			7	0.038	0.019	2.014	**0.048**
			8	0.079	0.025	3.113	**0.003**
		6	7	0.005	0.017	0.316	0.753
			8	0.046	0.023	1.964	*0.054*
		7	8	0.041	0.018	2.281	**0.026**

(Continued)

Table 1: continued

	Living condition	Captivity	Free-ranging	0.028	0.026	1.077	0.286
	Sampling date			0.0000004	0.0000966	0.004	0.997
	Storage duration			0.0000008	0.0000153	0.053	0.958
	Sampling time			0.00002	0.00003	0.490	0.626
	Time from sampling to storage			−0.00001	0.00001	−0.688	0.494
	Restraint duration			−0.00002	0.00001	−1.384	0.171
GPX	Sex	Female	Male	0.125	0.044	2.819	**0.006**
Units/mg proteins	Age class	5	6	0.011	0.072	0.148	0.882
			7	−0.047	0.064	−0.734	0.465
			8	−0.050	0.085	−0.586	0.560
		6	7	−0.058	0.056	−1.026	0.309
			8	−0.061	0.078	−0.772	0.443
		7	8	−0.003	0.060	−0.047	0.963
	Living condition	Captivity	Free-ranging	0.022	0.085	0.254	0.800
	Sampling date			0.0005	0.0003	1.407	0.164
	Storage duration			0.00019	0.00005	3.801	**<0.001**
	Sampling time			−0.00009	0.00010	−0.908	0.367
	Time from sampling to storage			0.00007	0.00005	1.408	0.164
	Restraint duration			−0.000005	0.000034	−0.131	0.896

not need to upregulate the activity of sod and gpx because the amount of damage generated was still within levels that cells can tolerate. Irrespective of the mechanisms underlying these responses, both markers of oxidative damage were sensitive to duration of restraint stress. This suggests that these markers of oxidative status might be reliable markers to detect the impact of acute exposure to environmental stressors on cheetahs. Further work to validate this hypothesis is needed.

Although restraint duration was an important predictor of some oxidative status markers, large individual variation occurred. Prior work revealed that individual variation in oxidative status markers may be explained by styles of coping (e.g. proactive vs. reactive) with stress source (Herborn *et al.*, 2011; Costantini *et al.*, 2012). One of the fundamental features that characterizes behavioural types is the responsiveness to environmental stimuli. Variation in the way individuals behaviourally cope with stressful episodes can be modelled along an axis polarized at the two extremes by proactive and reactive responses (Koolhaas *et al.*, 1999, 2010). When confronted to a challenging situation, such as a physical restraint, proactive individuals tend to respond with a strong sympathetic activation and increase in noradrenergic stimulation, resulting in a general fight-or-flight behavioural response (e.g. Carere *et al.*, 2010; Koolhaas *et al.*, 2010; Coppens *et al.*, 2010). In contrast, reactive individuals respond to a challenge with a strong hypothalamic-pituitary-adrenocortical reactivity (Carere *et al.*, 2010; Coppens *et al.*, 2010; Koolhaas *et al.*, 2010), resulting in a freezing response to stimulus and an increase in circulating glucocorticoids. Research on the links between coping style and oxidative status markers in cheetahs warrants further investigation in order to identify which characteristics make some individuals more vulnerable to environmental stressors.

Prior work on captive cheetahs, particularly in zoological gardens, revealed a high prevalence of degenerative and infectious diseases that cause morbidity and mortality, whereas free-ranging cheetahs do not exhibit such diseases (Munson *et al.*, 1999, 2005; Thalwitzer *et al.*, 2010; Terio *et al.*, 2012). Studies on domestic carnivores, such as dogs, found that markers of blood oxidative damage are higher in individuals with *Leishmaniasis* infection (Paltrinieri *et al.*,

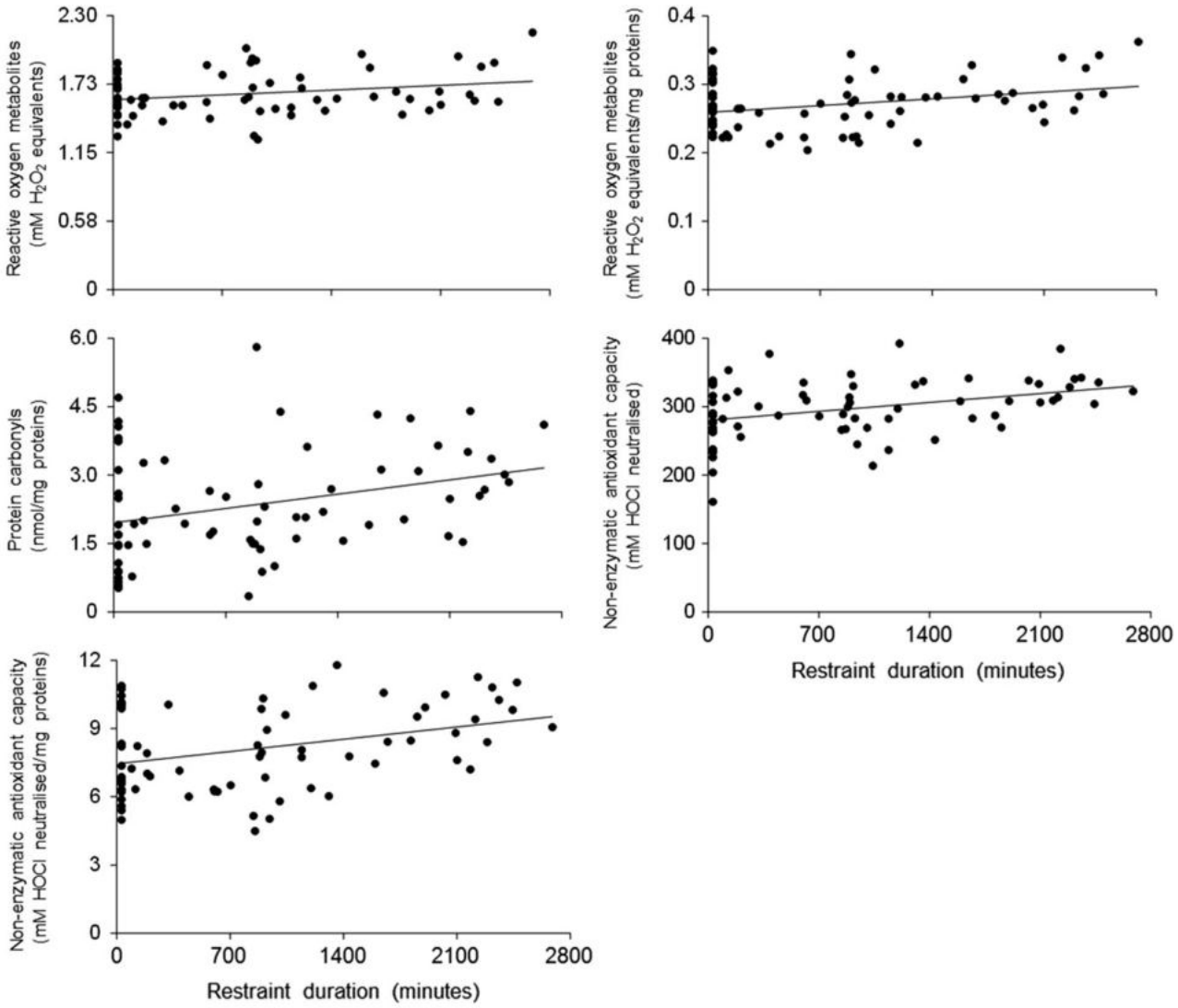

Figure 1: Cheetahs that were restraint for a longer duration had significantly higher levels of oxidative damage (reactive oxygen metabolites and protein carbonyls) and of serum non-enzymatic antioxidant capacity. Sample sizes of each age class were as follows: 68 for reactive oxygen metabolites; 70 for protein carbonyls and for antioxidant capacity

2010; Almeida *et al.*, 2013) or affected by lymphoma (Winter *et al.*, 2009) or mastocytoma (Finotello *et al.*, 2014), indicating that oxidative damage can be associated with a disease status in carnivores. Captive cheetahs in our study had higher levels of oxidative damage markers than free-ranging cheetahs, but did not show any overt clinical signs of diseases. In southern African countries, captive cheetahs are often fed with diets that consist primarily of lean muscle meat from cattle, horses or donkeys, supplemented with a multivitamin/mineral powder (Tordiffe *et al.*, 2016). A link between an unnatural diet composition and the pathogenesis of diseases has been suggested (e.g. Depauw *et al.*, 2012, 2013; Whitehouse-Tedd *et al.*, 2015). A comparison of the total serum fatty acid profiles between captive and free-ranging cheetahs in Namibia demonstrated that most of the polyunsaturated and monounsaturated fatty acids occur at significantly lower concentrations in the serum of free-ranging animals (Tordiffe *et al.*, 2016). A high intake of polyunsaturated fatty acids by captive cheetahs might contribute to the higher oxidative damage in these animals because polyunsaturated fatty acids may be quickly oxidized by free radicals (lipid peroxidation) generating many kinds of reactive oxygen metabolites (Halliwell and Gutteridge, 2015). Moreover, lipid peroxidation end-products, such as malondialdehyde, may cause carbonylation of proteins (Halliwell and Gutteridge, 2015), which might be one reason

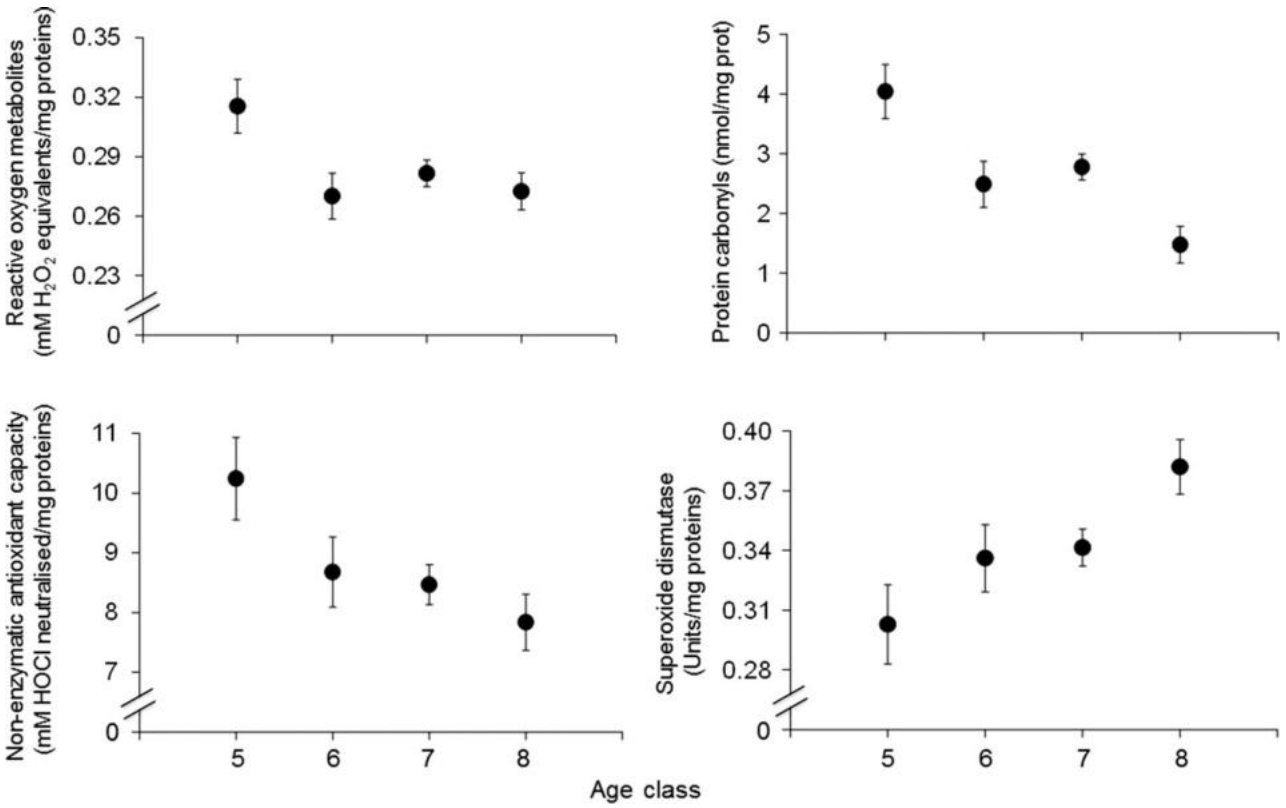

Figure 2: The serum non-enzymatic antioxidant capacity and both serum markers of oxidative damage (reactive oxygen metabolites standardised by protein concentration and protein carbonyls) were lower, while the activity of superoxide dismutase was higher in older cheetahs. Sample sizes of each age class were as follows: 9 (10 for sod) for age class 5; 13 for age class 6; 31 (reactive oxygen metabolites), 33 (protein carbonyls and antioxidant capacity) and 36 (sod) for age class 7; 15 for age class 8

for the higher amount of protein carbonyls we detected in captive cheetahs.

Our results demonstrate significant lower levels of oxidative damage markers and higher sod activity in older than younger cheetahs. Little is known about age-related changes in oxidative status markers in free-ranging animals. Studies on humans and laboratory mammals detected only moderate support for age-related changes in oxidative damage markers or antioxidants (Jacob *et al.*, 2013; Costantini, 2014). Most studies conducted so far have been cross-sectional, i.e. a comparison of age classes of individuals rather than within-individual changes over time in oxidative status markers (Costantini, 2014; Speakman *et al.*, 2015). Cross-sectional studies may be confounded by selective disappearance, if early mortality of poor-quality individuals occurs. If early mortality of those individuals having higher oxidative damage occurs, this would result in lower levels of oxidative damage in older age classes of individuals. A few studies on laboratory and wild animals have found evidence for selective disappearance of individuals having higher oxidative stress (Matsuo *et al.*, 1993; Alonso-Alvarez *et al.*, 2006; Herborn *et al.*, 2015; Marasco *et al.*, 2017), but also found evidence that age-related changes in oxidative status markers may be modulated by environmental conditions (Marasco *et al.*, 2017). Our cross-sectional data revealed a decline of oxidative damage markers and non-enzymatic antioxidants and an increase of sod with age. These results might suggest selective disappearance of younger cheetahs having more oxidative damage and less sod. Our data would be in line with a within-individual decline with age in levels of oxidative damage markers in free-ranging banded mongooses *Mungos mungo* (Vitikainen *et al.*, 2016). The low number of resampled individuals in this study did not allow us to provide definitive conclusions and stimulates further investigation on within-individual changes of oxidative status markers across life.

Cheetah males have a unique spatial system within mammalian species with adult males exhibiting two distinct spatial tactics. Males are either territorial and defend a small territory or they are non-territorial, i.e. floaters, and roam over large areas encompassing several territories of territory holders (Caro and Collins, 1987a,b; Melzheimer *et al.*, unpublished results). Floaters in the Serengeti National Park in Tanzania tend to have elevated serum cortisol concentrations and white blood cell counts and lower muscle mass, suggesting a poorer health status as compared to territory holders

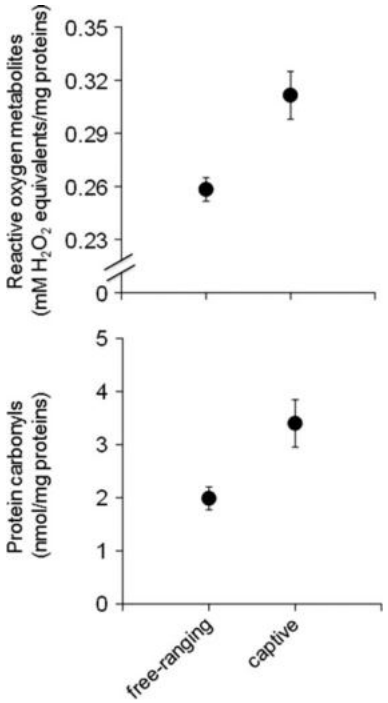

Figure 3: Captive cheetahs had significantly higher serum markers of oxidative damage (reactive oxygen metabolites standardised by protein concentration and protein carbonyls) than free-ranging cheetahs. Sample sizes of free-ranging and captive cheetahs were as follows: 48 and 20 for reactive oxygen metabolites, respectively; 50 and 20 for protein carbonyls, respectively

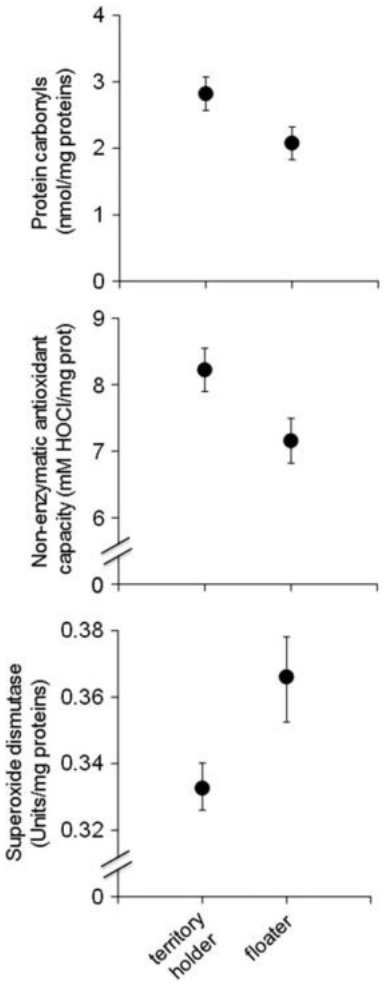

Figure 4: Territory holders had significant higher serum protein carbonyls and serum non-enzymatic micro-molecular antioxidant capacity and lower superoxide dismutase activity than floater males. Sample sizes of territory holders and floaters were as follows: 19 and 16 for serum protein carbonyls, respectively; 16 and 15 for non-enzymatic micro-molecular antioxidant capacity, respectively; 20 and 17 for superoxide dismutase, respectively

(Caro *et al.*, 1989). In our population, we found that territory holders had higher levels of oxidative damage markers to proteins and lower sod protection than floaters, suggesting hitherto unexplored metabolic costs of holding and defending a territory in cheetahs. For example, once cheetahs manage to take over a territory they start gaining body mass (Melzheimer *et al.*, unpublished results). This increase in body mass might be associated with increase protein turnover and metabolic activity, resulting in higher damage to proteins.

In recent years, there has been a growing interest in the role of oxidative stress as a cost of reproduction (reviewed in Stier *et al.*, 2012; Metcalfe and Monaghan, 2013; Costantini, 2014). Our data do not provide support for this hypothesis because females accompanied with their offspring and solitary females did not differ in any of the markers of oxidative status measured. Within females accompanied by offspring, however, those that were lactating tended to have lower oxidative damage than those with weaned offspring. Although the sample size was too small to conduct robust statistical analyses, our data on effect size estimates are in agreement with findings in mammals showing lower levels of oxidative damage in lactating than post-lactation females (Vitikainen *et al.*, 2016). It has been suggested that during specific phases of reproduction, protective mechanisms might be upregulated to protect offspring from direct (e.g. deposition of oxidised molecules in milk or yolk) or indirect (e.g. poor health state of the mother) negative effects of oxidative stress (Garratt *et al.*, 2011; Costantini, 2014; Blount *et al.*, 2016). However, our data show that lactating females also had lower rather than higher antioxidant defences (gpx activity and non-enzymatic antioxidant capacity) than post-lactation females. These results might indicate that the basal oxidative status changes across the phases of reproduction as a consequence of the numerous metabolic and hormonal adjustments that occur in reproducing females. These descriptive data on the link between reproductive phases and

Table 2: Descriptive statistics of solitary females, lactating and post-lactation females. Effect size estimates are shown as Pearson correlation coefficients and refer to the comparison between lactating and post-lactation females. Effect size was calculated using the compute.es package (Del Re, 2013) in R (R Core Team, 2013)

ROMs (mM)	2.88 ± 1.05 (7)	2.15 ± 0.27 (4)	3.11 ± 0.58 (4)	−0.73 [−0.97, 0.17]
ROMs (mM/mg proteins)	0.091 ± 0.037 (7)	0.053 ± 0.005 (4)	0.095 ± 0.020 (4)	−0.82 [−0.98, −0.06]
Protein carbonyls (nmol/mg proteins)	2.65 ± 1.01 (7)	1.61 ± 0.65 (4)	2.85 ± 0.99 (4)	−0.6 [−0.94, 0.39]
OXY (mM)	302 ± 38 (7)	320 ± 30 (4)	360 ± 36 (4)	−0.52 [−0.93, 0.48]
OXY (mM/mg proteins)	9.50 ± 1.90 (7)	7.91 ± 1.50 (4)	10.93 ± 1.49 (4)	−0.71 [−0.96, 0.20]
SOD (Units/mg proteins)	1.13 ± 0.10 (8)	1.38 ± 0.15 (4)	1.16 ± 0.13 (5)	0.61 [−0.25, 0.93]
GPX (Units/mg proteins)	0.405 ± 0.191 (8)	0.282 ± 0.165 (4)	0.504 ± 0.133 (5)	−0.60 [−0.93, 0.27]

CI, confidence interval.

blood oxidative status warrant further investigation to understand the role of oxidative stress in mediating female's choice of when to reproduce and how much to invest in lactation.

The time of day or year of blood sampling, the time elapsed from sampling to storage and storage duration were not significantly associated with any of the blood oxidative status markers analysed with the exception of gpx for storage duration. This is surprising particularly for the time elapsed from sampling to storage and storage duration because it is generally assumed that all markers of oxidative status degrade over time. However, some studies demonstrated both short-term stability (refrigeration at 4°C of blood samples for 24 h, Celi *et al.*, 2010; storage time between 3 h and 48 h at 4°C or 20°C, respectively, Jansen *et al.*, 2013) and long-term stability (21–24 months at −80°C, Abiaka *et al.*, 2000, Cavalleri *et al.*, 2004) of the markers we used. The gpx activity was, however, conversely to our expectation, higher rather than lower in older samples. Although our data do not exclude that some degradation may have occurred, it was minor as compared to the size of the biological effects.

In conclusion, we demonstrated that some blood-based markers of oxidative status are involved in the physiological responsiveness of cheetahs to short-term unpredictable environmental stressors. We also demonstrated that oxidative stress might be one additional mechanism underlying the detrimental effects of captivity on the health status of cheetahs. Further data on within-individual variation in markers of oxidative status are necessary to understand whether the lower levels of oxidative damage we detected in older individuals is a sign of either ageing or general decrease in body function. Finally, our data also stimulate further work on the role of oxidative stress as a mediator of spatial tactics and reproductive strategies in cheetahs.

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