

Socioecological and environmental predictors of physiological stress markers in a threatened feline species

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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 restrained 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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Introduction

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 (cortosterone 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.

Materials and methods

Sampling

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 restrained 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).

Laboratory analyses

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 H₂O₂ equivalents and as mM H₂O₂ 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 micro-molecular 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.

Statistical analyses

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.

Results

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 ($SD \pm 0.04$) for reactive oxygen metabolites ($n = 5$), -0.68 ($SD \pm 1.60$) for protein carbonyls ($n = 5$), -0.76 ($SD \pm 3.54$) for the non-enzymatic micro-molecular antioxidant capacity ($n = 5$) and $+0.16$ ($SD \pm 0.45$) 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).

Discussion

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 ecophysiologicals 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

Variable	Factor	Reference level	Level	Coefficient estimate	SE	<i>t</i>	<i>P</i>	
ROMs	Sex	Female	Male	0.070	0.054	1.299	0.199	
mM H ₂ O 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	8	0.055	0.069	0.795	0.430
				8	0.089	0.096	0.929	0.357
				8	0.035	0.074	0.471	0.639
				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 ₂ O 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	
		6	7	8	-0.009	0.013	-0.745	0.459
				8	-0.047	0.018	-2.615	0.012
				8	-0.00003	0.00006	-0.506	0.622
				8	-0.000007	0.0000103	-0.677	0.502
				8	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	8	0.288	0.399	0.723
8					-1.03	0.546	-1.878	0.066
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

Variable	Factor	Reference level	Level	Coefficient estimate	SE	t	P
	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

Variable	Factor	Reference level	Level	Coefficient estimate	SE	t	P
	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.*,

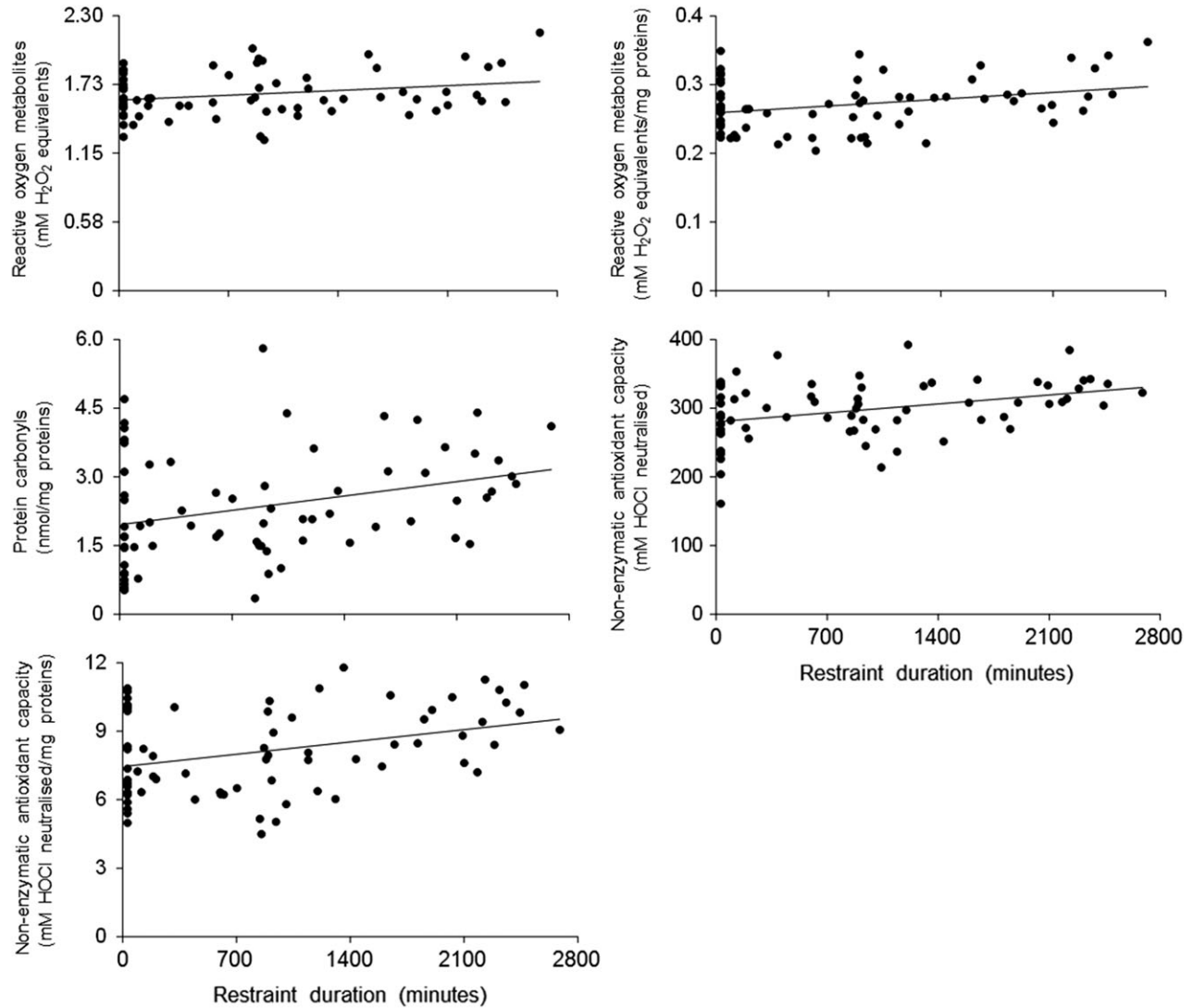


Figure 1: Cheetahs that were restraint for a longer duration had significantly higher levels of oxidative damage (reactive oxygen metabolites and protein carbonyls) and of serum non-enzymatic antioxidant capacity. Sample sizes of each age class were as follows: 68 for reactive oxygen metabolites; 70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

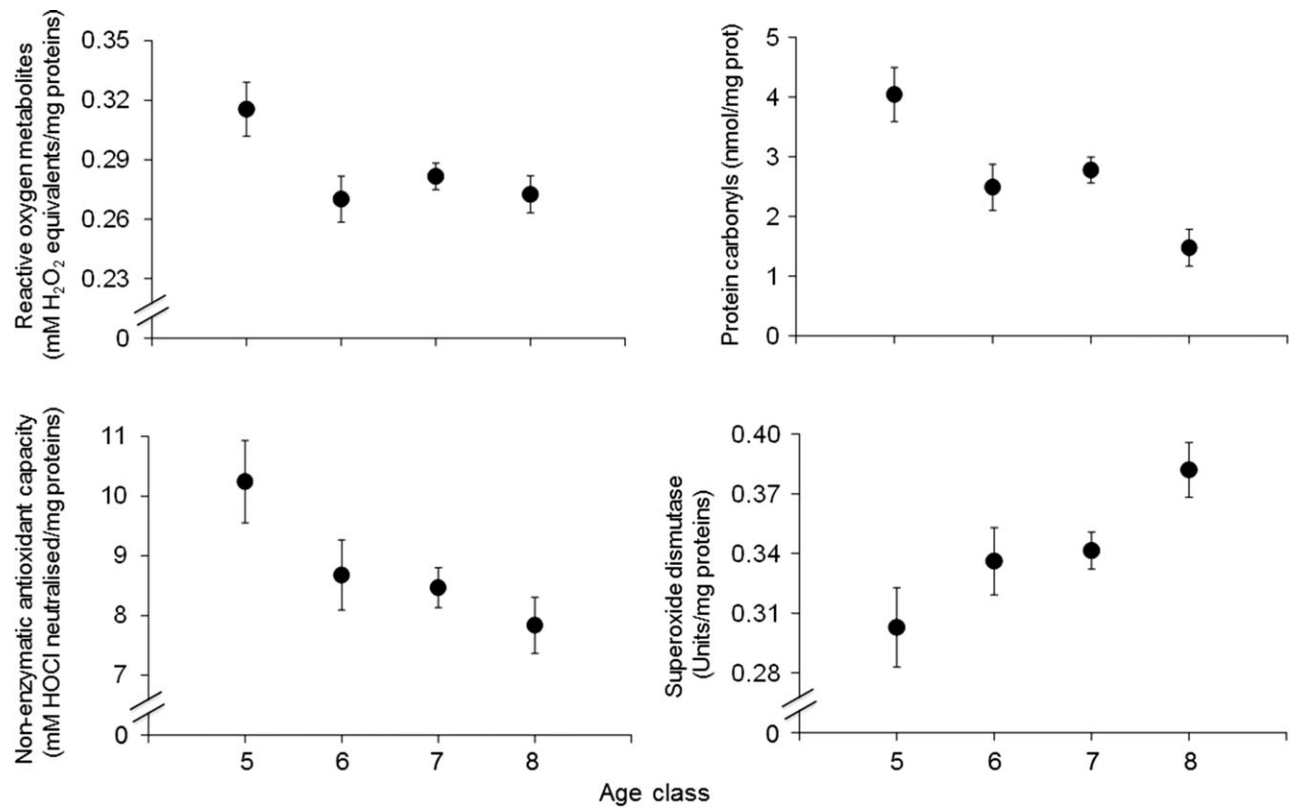


Figure 2: The serum non-enzymatic antioxidant capacity and both serum markers of oxidative damage (reactive oxygen metabolites standardised by protein concentration and protein carbonyls) were lower, while the activity of superoxide dismutase was higher in older cheetahs. Sample sizes of each age class 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

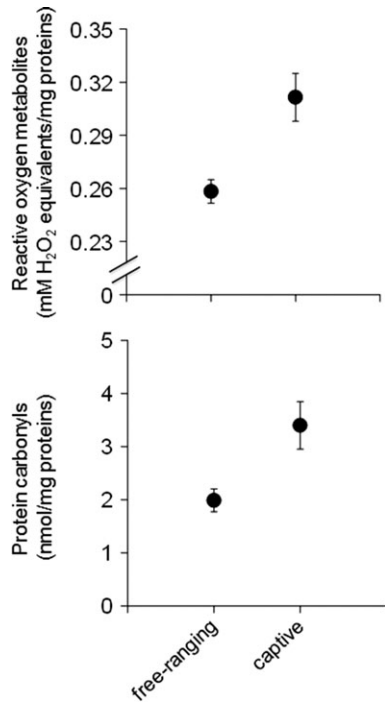


Figure 3: Captive cheetahs had significantly higher serum markers of oxidative damage (reactive oxygen metabolites standardised by protein concentration and protein carbonyls) than free-ranging cheetahs. Sample sizes of free-ranging and captive cheetahs were as follows: 48 and 20 for reactive oxygen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

(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

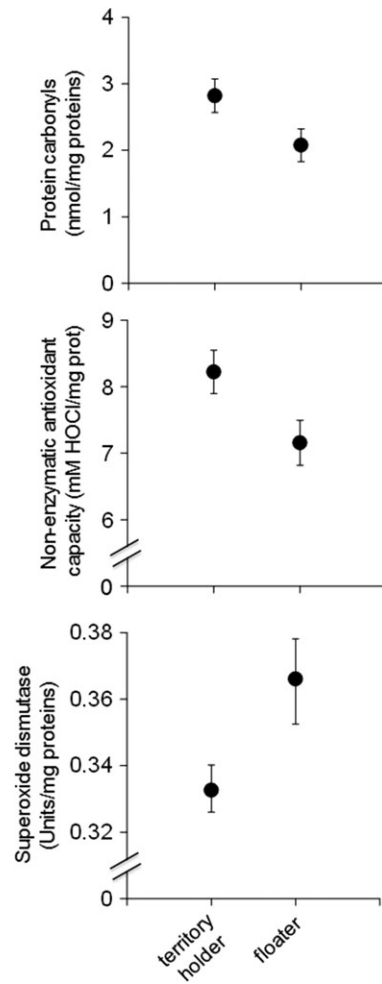


Figure 4: Territory holders had significant higher serum protein carbonyls and serum non-enzymatic micro-molecular antioxidant capacity and lower superoxide dismutase activity than floater males. Sample sizes of territory holders and floaters were as follows: 19 and 16 for serum protein carbonyls, respectively;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

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)

	Solitary	Lactation	Post-lactation	<i>r</i> [95 %CI]
	Mean ± SD (n)	Mean ± SD (n)	Mean ± SD (n)	
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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References

- Abiaka C, Al-Awadi F, Olusi S (2000) Effect of prolonged storage on the activities of superoxide dismutase, glutathione reductase, and glutathione peroxidase. *Clin Chem* 46:566–567.
- Almeida BFM, Narciso LG, Melo LM, Preve PP, Bosco AM, Lima VMF, Ciarlina PC (2013) Leishmaniasis causes oxidative stress and alteration of oxidative metabolism and viability of neutrophils in dogs. *Vet J* 198:599–605.
- Alonso-Alvarez C, Bertrand S, Devevey G, Prost J, Faivre B, Chastel O, Sorci G (2006) An experimental manipulation of life-history trajectories and resistance to oxidative stress. *Evolution* 60:1913–1924.

- Beaulieu M, Costantini D (2014) Biomarkers of oxidative status: missing tools in conservation physiology. *Conserv Physiol* 2:cou014. doi:10.1093/conphys/cou014.
- Beaulieu M, Thierry A-M, González-Acuña D, Polito MJ (2013) Integrating oxidative ecology into conservation physiology. *Conserv Physiol* 1:cot004. doi:10.1093/conphys/cot004.
- Blount JD, Vitikainen EI, Stott I, Cant MA (2016) Oxidative shielding and the cost of reproduction. *Biol Rev* 91:483–497.
- Busch DS, Hayward LS (2009) Stress in a conservation context: a discussion of glucocorticoid actions and how levels change with conservation-relevant variables. *Biol Conserv* 142:2844–2853.
- Carere C, Caramaschi D, Fawcett T (2010) Covariation between personalities and individual differences in coping with stress: converging evidence and hypotheses. *Curr Zool* 56:728–740.
- Caro TM (1994) *Cheetahs of the Serengeti Plains: Group Living in an Asocial Species*. University of Chicago Press, Chicago.
- Caro T, Collins D (1987a) Ecological characteristics of territories of male cheetahs (*Acinonyx jubatus*). *J Zool* 211:89–105.
- Caro T, Collins D (1987b) Male cheetah social organization and territoriality. *Ethology* 74:52–64.
- Caro TM, Fitzgibbon CD, Holt ME (1989) Physiological costs of behavioural strategies for male cheetahs. *Anim Behav* 38:309–317.
- Cavalleri A, Colombo C, Venturelli E, Miceli R, Mariani L, Cornelli U, Pala V, Berrino F, Secreto G (2004) Evaluation of reactive oxygen metabolites in frozen serum samples. Effect of storage and repeated thawing. *Int J Biol Markers* 18:250–253.
- Celi P, Sullivan M, Evans D (2010) The stability of the reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests on stored horse blood. *Vet J* 183:217–218.
- Cohen J (1988) *Power Analysis for the Behavioural Sciences*. Erlbaum, Hillsdale.
- Cooke SJ, Sack L, Franklin CE, Farrell AP, Beardall J, Wikelski M, Chown SL (2013) What is conservation physiology? Perspectives on an increasingly integrated and essential science. *Conserv Physiol* 1:cot001. doi:10.1093/conphys/cot001.
- Coppens CM, De Boer SF, Koolhaas JM (2010) Coping styles and behavioural flexibility: towards underlying mechanisms. *Phil Trans R Soc Lond B* 365:4021–4028.
- Costantini D (2008) Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol Lett* 11:1238–1251.
- Costantini D (2014) *Oxidative Stress and Hormesis in Evolutionary Ecology and Physiology*. Springer-Verlag, Berlin and Heidelberg.
- Costantini D, Monaghan P, Metcalfe NB (2011) Biochemical integration of blood redox state in captive zebra finches (*Taeniopygia guttata*). *J Exp Biol* 214:1148–1152.
- Costantini D, Ferrari C, Pasquaretta C, Cavallone E, Carere C, von Hardenberg A, Réale D (2012) Interplay between plasma oxidative status, cortisol and coping styles in wild alpine marmots, *Marmota marmota*. *J Exp Biol* 215:374–383.
- Dantzer B, Fletcher QE, Boonstra R, Sheriff MJ (2014) Measures of physiological stress: a transparent or opaque window into the status, management, and conservation of a species? *Conserv Physiol* 2:cou023. doi:10.1093/conphys/cou023.
- Del Re AC (2013) Compute.es: compute effect sizes. R package version 0.2–2. <http://cran.r-project.org/web/packages/compute.es>.
- Depauw S, Hesta M, Whitehouse-Tedd K, Stagegaard J, Buyse J, Janssens G (2012) Blood values of adult captive cheetahs (*Acinonyx jubatus*) fed either supplemented beef or whole rabbit carcasses. *Zoo Biol* 31:629–641.
- Depauw S, Hesta M, Whitehouse-Tedd K, Vanhaecke L, Verbrugghe A, Janssens G (2013) Animal fibre: the forgotten nutrient in strict carnivores? First insights in the cheetah. *J Anim Physiol Anim Nutr* 97:146–154.
- Durant SM, Mitchell N, Groom R, Pettorelli N, Ipavec A, Jacobson AP, Woodroffe R, Böhm M, Hunter LTB, Becker MS, et al (2016) The global decline of cheetah (*Acinonyx jubatus*) and what it means for conservation. *Proc Natl Acad Sci USA* 114:528–533.
- Finotello R, Pasquini A, Meucci V, Lippi I, Rota A, Guidi G, Marchetti V (2014) Redox status evaluation in dogs affected by mast cell tumour. *Vet Comp Oncol* 12:120–129.
- French SS, Neuman-Lee LA, Terletzky PA, Kiriazis NM, Taylor EN, DeNardo DF (2017) Too much of a good thing? Human disturbance linked to ecotourism has a “dose-dependent” impact on innate immunity and oxidative stress in marine iguanas, *Amblyrhynchus cristatus*. *Biol Conserv* 210:37–47.
- Garratt M, Vasilaki A, Stockley P, McArdle F, Jackson M, Hurst JL (2011) Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. *Proc R Soc Lond B* 278:1098–1106.
- Georgiev AV, Thompson ME, Mandalaywala TM, Maestriepieri D (2015) Oxidative stress as an indicator of the costs of reproduction among free-ranging rhesus macaques. *J Exp Biol* 218:1981–1985.
- Glavin GB, Paré WP, Sandbak T, Bakke HK, Murison R (1994) Restraint stress in biomedical research: an update. *Neurosci Biobehav Rev* 18:223–249.
- Halliwell BH, Gutteridge JMC (2015) *Free Radicals in Biology and Medicine*. Oxford University Press, Oxford.
- Hau M, Haussmann MF, Greives TJ, Matlack C, Costantini D, Quetting M, Adelman JS, Miranda AC, Partecke J (2015) Repeated stressors in adulthood increase the rate of biological ageing. *Front Zool* 12:4. doi:10.1186/s12983-015-0095-z.
- Helfenstein F, Losdat S, Möller AP, Blount JD, Richner H (2010) Sperm of colourful males are better protected against oxidative stress. *Ecol Lett* 13:213–222.
- Herborn KA, Coffey J, Larcombe SD, Alexander L, Arnold KE (2011) Oxidative profile varies with personality in European greenfinches. *J Exp Biol* 214:1732–1739.

- Herborn KA, Daunt F, Heidinger BJ, Granroth-Wilding HMV, Burthe SJ, Newell MA, Monaghan P (2015) Age, oxidative stress exposure and fitness in a long-lived seabird. *Funct Ecol* 30:913–921.
- Isaksson C, Sheldon BC, Uller T (2011) The challenges of integrating oxidative stress into life-history biology. *Bioscience* 61:194–202.
- Isaksson C, Sepil I, Baramidze V, Sheldon BC (2013) Explaining variance of avian malaria infection in the wild: the importance of host density, habitat, individual life-history and oxidative stress. *BMC Ecol* 13: 1–11. doi:10.1186/1472-6785-13-15.
- Jacob KD, Noren Hooten N, Trzeciak AR, Evans MK (2013) Markers of oxidant stress that are clinically relevant in aging and age-related disease. *Mech Ageing Dev* 134:139–157. doi:10.1016/j.mad.2013.02.008.
- Jansen EHJM, Beekhof PK, Cremers JWJM, Vezeliene D, Muzakova V, Skalicky J (2013) Short-term stability of biomarkers of oxidative stress and antioxidant status in human serum. *ISRN Biomarkers* 2013:316528. doi:10.1155/2013/316528.
- Koolhaas JM, Korte SM, de Boer SF, van der Vegt BJ, van Reenen CG, Hopster H, de Jong IC, Ruis MAW, Blokhuis HJ (1999) Coping styles in animals: current status in behavior and stress-physiology. *Neurosci Biobehav Rev* 23:925–935.
- Koolhaas JM, De Boer SF, Coppens CM, Buwalda B (2010) Neuroendocrinology of coping styles: towards understanding the biology of individual variation. *Front Neuroendocrinol* 31:307–321.
- Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186:464–478.
- Marasco V, Stier A, Boner W, Griffiths K, Heidinger B, Monaghan P (2017) Environmental conditions can modulate the links among oxidative stress, age, and longevity. *Mech Ageing Dev* 164:100–107.
- Matsuo M, Gomi F, Kuramoto K, Sagai M (1993) Food restriction suppresses an age-dependent increase in the exhalation rate of pentane from rats: a longitudinal study. *J Gerontol* 48:B133–B138.
- McEwen BS, Stellar E (1993) Stress and the individual—mechanisms leading to disease. *Arch Inter Med* 153:2093–2101.
- Metcalfe NB, Monaghan P (2013) Does reproduction cause oxidative stress? An open question. *Trends Ecol Evol* 28:347–350.
- Montoya B, Valverde M, Rojas E, Torres R (2016) Oxidative stress during courtship affects male and female reproductive effort differentially in a wild bird with biparental care. *J Exp Biol* 219:3915–3926.
- Munck A, Guyre PM, Holbrook NJ (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr Rev* 5:25–44.
- Munson L, Nesbit JW, Meltzer DG, Colly LP, Bolton L, Kriek NP (1999) Diseases of captive cheetahs (*Acinonyx jubatus jubatus*) in South Africa: a 20-year retrospective survey. *J Zoo Wildl Med* 30:342–347.
- Munson L, Terio KA, Worley M, Jago M, Bagot-Smith A, Marker L (2005) Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. *J Wildl Dis* 41:542–548.
- Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 70:158–169.
- Paltrinieri S, Ravicini S, Rossi G, Roura X (2010) Serum concentrations of the derivatives of reactive oxygen metabolites (d-ROMs) in dogs with leishmaniosis. *Vet J* 186:393–395.
- Pap PL, Vágási CI, Czirájk GÁ, Titilincu A, Pinteá A, Osváth G, Fülöp A, Barta Z (2011) The effect of coccidians on the condition and immune profile of molting house sparrows (*Passer domesticus*). *Auk* 128:330–339.
- R Core Team, et al.2013R: A language and environment for statistical computing. R Foundation for Statistical Computing Vienna, Austria. <https://www.R-project.org/>
- Romero LM (2004) Physiological stress in ecology: lessons from biomedical research. *Trends Ecol Evol* 19:249–255.
- Romero LM, Dickens MJ, Cyr NE (2009) The reactive scope model—a new model integrating homeostasis, allostasis, and stress. *Horm Behav* 55:375–389.
- Saino N, Caprioli M, Romano M, Boncoraglio G, Rubolini D, Ambrosini R, Bonisoli-Alquati A, Romano A (2011) Antioxidant defenses predict long-term survival in a passerine bird. *PLoS One* 6:e19593. doi:10.1371/journal.pone.0019593.
- Sebastiano M, Eens M, Ablelgawad H, de Thoisy B, Lacoste V, Pineau K, Asard H, Chastel O, Costantini D (2017) Oxidative stress biomarkers are associated with visible clinical signs of a disease in frigatebird nestlings. *Sci Rep* 7:1599. doi:10.1038/s41598-017-01417-9.
- Semeniuk CAD, Bourgeon S, Smith SL, Rothley KD (2009) Hematological differences between stingrays at tourist and non-visited sites suggest physiological costs of wildlife tourism. *Biol Conserv* 142:1818–1829.
- Speakman JR, Blount JD, Bronikowski AM, Buffenstein R, Isaksson C, Kirkwood TBL, Monaghan P, Ozanne SE, Beaulieu M, Briga M, et al (2015) Oxidative stress and life histories: unresolved issues and current needs. *Ecol Evol* 5:5745–5757.
- Stier A, Reichert S, Massemin S, Bize P, Criscuolo F (2012) Constraint and cost of oxidative stress on reproduction: correlative evidence in laboratory mice and review of the literature. *Front Zool* 9:37. doi:10.1186/1742-9994-9-37.
- Terio KA, Munson L, Moore PF (2012) Characterization of the gastric immune response in cheetahs (*Acinonyx jubatus*) with Helicobacter-associated gastritis. *Vet Pathol* 49:824–833.
- Thalwitzer S, Wachter B, Robert N, Wibbelt G, Müller T, Lonzer J, Meli ML, Bay G, Hofer H, Lutz H (2010) Seroprevalences to viral pathogens in free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland. *Clin Vaccine Immunol* 7: 232–238.

- Tordiffe ASW, Wachter B, Heinrich SK, Reyers F, Mienie LJ (2016) Comparative serum fatty acid profiles of captive and free-ranging cheetahs (*Acinonyx jubatus*) in Namibia. *PLoS One* 11:e0167608. doi:10.1371/journal.pone.0167608.
- Vitikainen EIK, Cant MA, Sanderson JL, Mitchell C, Nichols HJ, Marshall HH, Thompson FJ, Gilchrist JS, Hodge SJ, Johnstone RA, et al (2016) Evidence of oxidative shielding of offspring in a wild mammal. *Front Ecol Evol* 4:58. doi:10.3389/fevo.2016.00058.
- Whitehouse-Tedd KM, Lefebvre SL, Janssens GPJ (2015) Dietary factors associated with faecal consistency and other indicators of gastrointestinal health in the captive cheetah (*Acinonyx jubatus*). *PLoS One* 10:e0120903. doi:10.1371/journal.pone.0120903.
- Wingfield JC (2013) The comparative biology of environmental stress: behavioural endocrinology and variation in ability to cope with novel, changing environments. *Anim Behav* 85:1127–1133.
- Wingfield JC, Maney DL, Breuner CW, Jacobs JD, Lynn S, Ramenofsky M, Richardson RD (1998) Ecological bases of hormone–behavior interactions: the “emergency life history stage”. *Am Zool* 38:191–206.
- Winter JL, Barber LG, Freeman L, Griessmayr PC, Milbury PE, Blumberg JB (2009) Antioxidant status and biomarkers of oxidative stress in dogs with lymphoma. *J Vet Intern Med* 23:311–316.
- Zaidi SM, Al-Qirim TM, Hoda N, Banu N (2003) Modulation of restraint stress induced oxidative changes in rats by antioxidant vitamins. *J Nutr Biochem* 14:633–636.