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Sex and species differences of stress markers in sympatric cheetahs and leopards in Namibia

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ABSTRACT

Physiological stress markers may provide valuable insight for our understanding of costs of given life-history strategies or of wildlife health condition, most importantly in case of threatened species. In the last decade, there has been growing interest in the ecological relevance of cellular oxidative stress, which would provide complimentary information to that obtained by the classic analyses of glucocorticoid hormones. In this study, we analysed the sex and species variation of five blood-based markers of oxidative status, both molecular oxidative damage and antioxidant protection, in sympatric cheetahs (*Acinonyx jubatus*) and leopards (*Panthera pardus*) living on Namibian farmlands. Both these terrestrial carnivores are classified as vulnerable by the International Union for Conservation of Nature. We found that female cheetahs had significantly higher serum reactive oxygen metabolites of non-protein origin and lower glutathione peroxidase activity in whole blood than both male and female leopards and male cheetahs. We also found that cheetahs and leopards differed in the association between the two antioxidant enzymes glutathione peroxidase and superoxide dismutase. Correlations among oxidative status markers were stronger in female cheetahs than leopards or male cheetahs. Our results suggest that female cheetahs are more sensitive to local sources of stress. Our work did not corroborate the assumption that two species with different life histories consistently differ in key physiological traits.

1. Introduction

Measures of physiological stress are commonly used to assess the costs of given life-history events and the health status of free-living animals. Glucocorticoids are valuable biomarkers to assess the stress response of individuals (Romero, 2004; Angelier and Wingfield, 2013; Dantzer et al., 2014) and the population health in ecological and conservation studies (Busch and Hayward, 2009; Dantzer et al., 2014). However, glucocorticoids do not provide a direct quantification of actual physiological costs (e.g., cellular damage) an organism accrues when being exposed to a stressor or demanding activity. Thus, in the last decade, there has been a burgeoning of studies that tested the connections between oxidative stress and environmental stressors or key life-history functions in free-ranging animals (reviewed in Costantini, 2014). Body functions like aerobic metabolism and activity of immune cells are responsible for the production of several reactive oxygen chemicals (e.g. free radical or non-free radical chemicals such as superoxide anion, hydrogen peroxide, hypochlorous acid or peroxynitrite). While these reactive oxygen chemicals are counteracted by the antioxidant system (enzymatic and non-enzymatic), they cause oxidation of proteins, lipids and nucleic acids (Halliwell and Gutteridge, 2015). The resulting chemical modifications of biomolecules caused by the reactive chemicals are referred as molecular oxidative damage, which is classically being used as an endpoint to estimate the oxidative stress level (Halliwell and Gutteridge, 2015). Eco- and conservation physiologists have become interested in oxidative stress, recognizing that the mechanisms needed to keep oxidative damage under control may underlie adaptability of species to environmental stressors and many life-history trade-offs (Costantini, 2008, 2014; Isaksson et al., 2011; Stier et al., 2012; Beaulieu and Costantini, 2014; Speakman et al., 2015; Alonso-Alvarez et al., 2017). There is considerable evidence that closely related species may significantly differ in many aspects of oxidative stress physiology, indicating low phylogenetic signals for some oxidative status markers (Cohen et al., 2008; Cohen and McGraw, 2009;

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Costantini, 2014). Other factors might therefore be more important in generating within and between species variation in makers of oxidative status. Studies carried out on free-ranging animals demonstrated significant links between oxidative status markers and key health and fitness related traits or environmental factors. For example, higher values of some blood oxidative damage markers were associated with reduced sperm quality (Helfenstein et al., 2010), high reproductive effort (Georgiev et al., 2015), reduced survival and reproductive output (Vitikainen et al., 2016), longer duration of restraint stress (Costantini et al., 2017), human disturbance stemming from ecotourism (Semeniuk et al., 2009; French et al., 2017), or sex (Costantini, 2018). Despite the emerging interest in antioxidants and oxidative stress ecology and the wealth of information that exists, there are still many open questions. For example, most of the literature is focussed on birds, with much less work having been done on other vertebrates (reviewed in Costantini, 2014). There is also very little work about the causes and consequences of individual variation in markers of oxidative status in threatened species. Knowledge of the variability of stress markers and of its predictors in threatened species is important for understanding and predicting the impact of ongoing environmental changes on population viability and for planning sustainable and successful conservation strategies.

In this study, we have measured five blood-based markers of oxidative status in sympatric cheetahs (*Acinonyx jubatus*) and leopards (*Panthera pardus*) living on Namibian farmlands, and have compared the values between the species and the sexes. Both carnivore species are classified as vulnerable by the International Union for Conservation of Nature (IUCN, 2018). We have used both univariate and multivariate statistical analyses because univariate analyses provide information on each single marker, whereas multivariate analyses extract a multivariate signal from all the analysed markers, which depends on the strength and direction to which markers associate with each other.

2. Materials and methods

2.1. Ethics statement

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, 2067/2015). Samples were transported once a year to Germany in full compliance with the Convention on International Trade in Endangered Species (CITES) and stored at −80 °C until laboratory analysis.

2.2. Sampling

Blood samples of 29 free-ranging cheetahs (17 males and 12 females) and 25 leopards (11 males and 14 females) were collected between 2012 and 2016 on commercial farmland in east-central Namibia (21°45′ S to 22°45′ S and 16°30′ E to 18°30′ E). Data on markers of oxidative status of 53 cheetahs have been previously published (Costantini et al., 2017). Here, we used a subset ($n = 29$) of that database to create a comparable dataset to leopards, i.e., cheetahs and leopards did not differ in sampling date, sampling time, restraint duration, age class, time from sampling to storage and storage duration (t-test, all $p \geq .12$). We previously showed that storage duration did not affect our results (Costantini et al., 2017).

Animals 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, animals 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 them. Cheetahs and leopards were immobilised by remote intramuscular injection using a dart gun. Cheetahs were given 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), while leopards were injected with 0.07 mg/kg medetomidine and 3.7 mg/kg ketamine. Blood samples were taken between 20 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 to 60 min, the animals were given an antidote (0.11 mg/kg atipamezole for cheetahs and 0.13 mg/kg for leopards; 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, USA) and transported to the laboratory at the field station in a cooler box. At the field station laboratory, non-heparinised tubes were spun to separate serum from blood clots. Serum and whole EDTA blood samples were stored in liquid nitrogen in Namibia.

2.3. Laboratory analyses

We have quantified five commonly used blood-based markers of oxidative status (e.g. Costantini et al., 2013, 2017; Vitikainen et al., 2016; French et al., 2017). Serum reactive oxygen metabolites, a marker of intermediate oxidative damage generated early in the oxidative cascade, were measured in duplicate using the d-ROMs assay (Diacron International, Grosseto, Italy) and 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, a marker of oxidative damage to proteins, were measured in duplicate using the Protein Carbonyl Colorimetric assay (Cayman Chemical Company, Ann Arbor, MI, USA) and values were expressed as nmol per mg of proteins. The serum non-enzymatic antioxidant capacity was measured in duplicate using the OXY-Adsorbent test (Diacron International). Values were expressed as mM of HOCl neutralised 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 protein antioxidants (i.e. non-enzymatic micro-molecular antioxidant capacity). The activity in whole blood of the enzyme superoxide dismutase (SOD), which prevents oxidation due to superoxide radical, was measured in duplicate using the Ransod assay (RANDOX Laboratories, Crumlin, UK) and was expressed as units of SOD per mg of proteins. The activity in whole blood of the enzyme glutathione peroxidase (GPx), which prevents oxidation due to hydrogen peroxide and organic hydroperoxides, was measured in duplicate using the Ransel assay (RANDOX Laboratories, Crumlin, UK) and was expressed as units of GPx per mg of proteins. The Bradford protein assay (Bio-Rad Laboratories, Hercules, 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.

2.4. Statistical analyses

General linear models (GLMs) were used to assess relationships between each oxidative status marker and the predictor variables species and sex as well as their interaction. GLMs were run using the software STATISTICA 10 (StatSoft. Inc., Tulsa, OK, USA). We then performed the Kaiser–Meyer–Olkin (KMO) test of sampling adequacy and Bartlett's test of sphericity to evaluate whether the dataset was appropriate for Factor Analysis. The KMO test quantifies whether the partial correlations among variables are strong enough (≥ 0.5) to support the use of Factor Analysis. Bartlett's test evaluates the null hypothesis that the correlation matrix is an identity matrix (i.e., each variable correlates perfectly with itself, but has no correlation with the other variables) – rejection of the null hypothesis indicates that the variables are sufficiently interrelated to justify Factor Analysis. Both

tests showed that the dataset was adequate (KMO test = 0.64; Bartlett's test = 48.1, $p < .0001$), thus a Factor Analysis with Principal Components Analysis (PCA) as extraction method was performed using the whole dataset for both species and the correlation matrix between the different markers of oxidative status to characterise the variability of each group, i.e. male and female cheetahs, and male and female leopards. Loadings of each marker on the main axes were reported as Pearson correlation coefficient when the correlation was significant. The integration of blood oxidative status markers (i.e. the strength of correlations) was estimated using the relative standard deviation of eigenvalues ($SD_{rel}(\lambda) = \sqrt{Var(\lambda)}/\sqrt{N-1}$) of the correlation matrix according to Pavlicev et al. (2009), Haber (2011) and Costantini et al. (2013). It is calculated as the ratio of the square root of the variance of eigenvalues (λ) to the square root of the number of variables (N) minus 1, and varies between zero (corresponding to no correlation between the variables) and one (perfect correlation between all variables). Eigenvalues were calculated by running a PCA on the correlation matrix for each group (i.e. male and female cheetahs, and male and female leopards), separately. GLMs were then used to assess whether the new combined variables generated by PCA differed between species and sexes. Finally, a Multivariate Analysis of Variance (MANOVA) was used to assess whether the correlation matrices between markers differed among the above-mentioned groups. This was accomplished by the fact that the MANOVA creates new dependent variables that maximize group differences. The new dependent variables are linear combinations of the measured dependent variables, thus they depend on the correlations between them. Both PCA and MANOVA were run using PAST version 1.94 (Hammer et al., 2001).

3. Results

GLMs detected significant interactions between species and sex for reactive oxygen metabolites of non-protein origin, for micro-molecular non-enzymatic antioxidant capacity of non-protein origin and for activity of GPx (Table 1). Post-hoc analyses showed that (1) female cheetahs had significantly higher reactive oxygen metabolites of non-protein origin than female leopards and male cheetahs (Fig. 1B), (2) female cheetahs and male leopards had significantly higher micro-molecular non-enzymatic antioxidant capacity of non-protein origin than male cheetahs (Fig. 2B) and (3) female cheetahs had significantly lower GPx activity than both male and female leopards and male cheetahs (Fig. 2C). All other GLMs did not reveal any significant effect of either species or sex, apart a significant difference in GPx between species (Table 1).

Table 1
Outcomes of general linear models used to detect the significant predictors of blood oxidative status markers. Significant *P*-values are shown in bold. The removal of the interaction when non-significant did not change the outcomes.

Variable	Factor	F	P
ROMs	Species	2.52	0.119
mM H_2O_2 equivalents	Sex	0.03	0.866
	Species*Sex	0.46	0.501
ROMs	Species	1.31	0.258
mM H_2O_2 equivalents/mg proteins	Sex	1.98	0.166
	Species*Sex	6.03	**0.018**
Protein carbonyls	Species	3.31	0.075
nmol/mg proteins	Sex	0.82	0.371
	Species*Sex	0.14	0.714
OXY	Species	0.40	0.532
mM HOCl neutralised	Sex	0.001	0.973
	Species*Sex	1.15	0.288
OXY	Species	0.58	0.451
mM HOCl neutralised/mg proteins	Sex	1.45	0.235
	Species*Sex	6.45	**0.014**
SOD	Species	1.31	0.258
Units/mg proteins	Sex	0.04	0.839
	Species*Sex	0.33	0.568
GPx	Species	4.94	**0.031**
Units/mg proteins	Sex	2.96	0.091
	Species*Sex	5.47	**0.023**
PC1	Species	0.54	0.466
	Sex	0.67	0.417
	Species*Sex	4.99	**0.030**
PC2	Species	4.66	**0.036**
	Sex	1.07	0.301
	Species*Sex	2.04	0.159

The first two principal components of the PCA explained 65.6% of the variance. The first principal component (PC1; 43.9% of the total variance) was significantly related to (from lower to higher values) higher reactive oxygen metabolites of non-protein origin (loading = 0.65), protein carbonyls (loading = 0.77), micro-molecular non-enzymatic antioxidant capacity (loading = 0.88) and lower SOD (loading = −0.59). The second principal component (PC2; 21.7% of the total variance) was related to (from lower to higher values) higher GPx (loading = 0.90) and lower SOD (loading = −0.51). The plot of the first two principal components (Fig. 3) showed strong overlap of the multivariate spaces among all leopards and male cheetahs, while female cheetahs showed a different multivariate space characterized by lower variability due to stronger correlations (i.e. higher integration coefficient) between markers. Integration indices were 0.54 for male cheetahs, 0.74 for female cheetahs and 0.38 for both male and female leopards. A GLM with PC1 as a dependent variable detected a significant interaction between species and sex (Table 1). Post-hoc analyses revealed that female cheetahs and male leopards had significantly higher values of PC1 than male cheetahs (Fig. 4a). A GLM with PC2 as a dependent variable detected a significant difference between species (Table 1), with leopards having higher values than cheetahs (Fig. 4b). The graphical results of the PCA plot were further supported by a MANOVA (Wilks' lambda; 0.451, $p = .0013$), which showed that female cheetahs differed significantly from both female (Hotelling p-value = .0022) and male (Hotelling p-value = .0133) leopards and from male cheetahs (Hotelling p-value = .0018).

4. Discussion

This is the first study that has explored the species and sex differences in multiple markers of oxidative status in two large free-ranging terrestrial carnivore species. Our univariate models detected sex differences within and between species at specific markers. The multivariate analyses found further sex differences that the univariate analyses did not detect. PCA indicated that female cheetahs had higher oxidative stress than the other groups and that the species differed in the covariation between GPx and SOD in red blood cells. Between-species differences cannot be attributed to different blood sampling and storage ($p \geq .12$) or plasma concentration of proteins ($p = .51$) used to standardize markers because they were similar between cheetahs and leopards.

One reason for the higher reactive oxygen metabolites (of non-protein origin) and lower GPx activity of female cheetahs compared to female leopards might lie in their lifetime cumulative reproductive costs rather than their current reproductive status. Cheetah females may have up to six cubs per reproductive event, whereas leopards have only one or two cubs (Kingdon et al., 2013). Thus, cheetah females face a higher reproductive effort over their lifetime. Concerning the reproductive status at sample collection, all female leopards included in our study were non-reproducing, while six out of 12 female cheetahs were lactating or together with weaned cubs. In our previous work, we found that the differences between solitary female and lactating or post-lactating female cheetahs in oxidative status markers were rather moderate, indicating that the higher oxidative stress of female cheetahs might not be explained by their current reproductive status (Costantini et al., 2017). It might be that cheetahs were being exposed to stress before they were captured. This is possible because box traps for cheetahs were set at marking trees in the core area of territorial males.

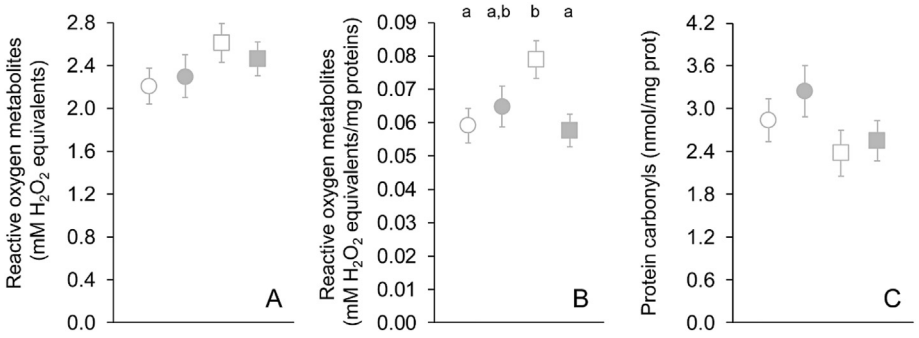

Fig. 1. Boxplots showing least square means ± standard error of oxidative damage markers. Means that are not sharing a same superscript (letters a and b) are significantly different from each other ($P < .05$). Note that superscripts are reported only in those cases where post-hoc tests were run. White dot = female leopard, grey dot = male leopard, white square = female cheetah, grey square = male cheetah.

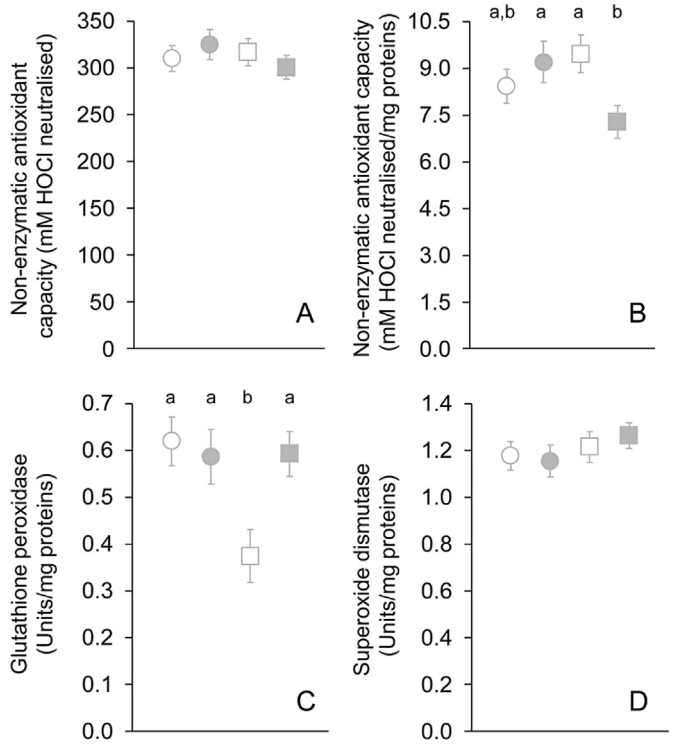

Fig. 2. Boxplots showing least square means ± standard error of both non-enzymatic and enzymatic antioxidant markers. Means that are not sharing a same superscript (letters a and b) are significantly different from each other ($P < .05$). Note that superscripts are reported only in those cases where post-hoc tests were run. White dot = female leopard, grey dot = male leopard, white square = female cheetah, grey square = male cheetah.

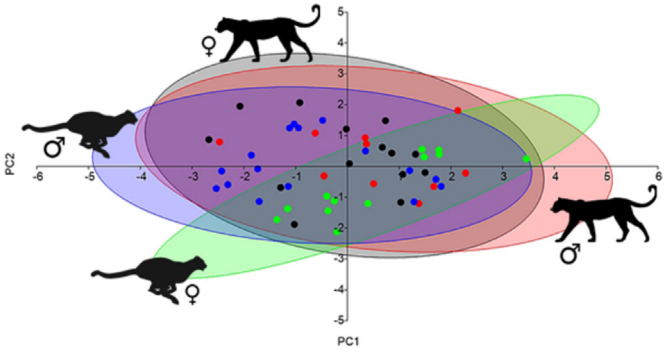

Fig. 3. Plot of the first two principal components showing a strong overlap of the multivariate spaces among all leopards and male cheetahs, while female cheetahs have a different multivariate space characterized by lower variability in the oxidative profile. Black dot = female leopard, red dot = male leopard, green dot = female cheetah, blue dot = male cheetah. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

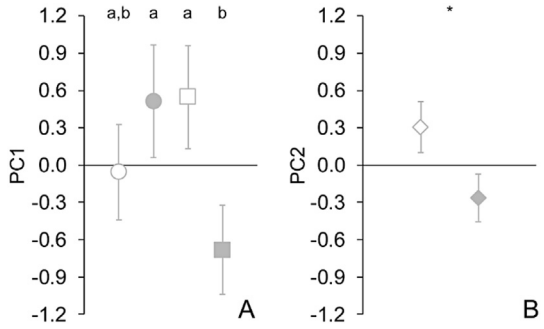

Fig. 4. Boxplots showing least square means ± standard error of PC1 and PC2. Means that are not sharing a same superscript (letters a and b) are significantly different from each other (P < .05). Note that superscripts are reported only in those cases where post-hoc tests were run. * indicates $P < .05$. White dot = female leopard, grey dot = male leopard, white square = female cheetah, grey square = male cheetah, white diamond = leopard, grey diamond = cheetah.

Female cheetahs enter in these core areas rarely and when they do so, they might encounter the territory holders (Caro, 1994). Such encounters are likely to be stressful for females because males are often aggressive towards females (Caro, 1994). Thus, it is probable that females are under stress when they are in territories of males. When facing with predictable or unpredictable events, organisms develop a suite of behavioral and physiological adjustments to maintain their homeostasis (Wingfield et al., 1998; Romero, 2004; Angelier and Wingfield, 2013). In particular, the hypothalamus-pituitary-adrenal axis plays an important role in the homeostasis maintenance through the release of glucocorticoid hormones. Glucocorticoids induce a number of metabolic modifications in the organism, including changes in the expression of antioxidant genes and in the activity of antioxidant enzymes or in the generation of oxidative damage that can be detectable within hours (Lin et al., 2004; Costantini et al., 2011).

Prior work on the same study populations of cheetahs and leopards revealed that the serum cortisol concentration after trapping was similar between male and female leopards, but was significantly higher in female than in male cheetahs (Heinrich et al., 2017). This result might indicate that female cheetahs are more sensitive to sources of stress than male cheetahs. However, the serum cortisol concentration we measured in cheetahs and leopards (Heinrich et al., 2017) provides a short-term view about the stress reactivity of animals while being restrained and does not provide any clue about cortisol levels before being caught. Nevertheless, female cheetahs might have been under higher long-term stress than the other sex and species groups, because of the high strength of correlations among the measured oxidative status markers, which significantly increase when the organism is being exposed to both a short- and long-term stressful situation (Dotan et al., 2004).

Our data also revealed differences between cheetahs and leopards in the association of the two enzymes GPx and SOD. The higher values of PC2 of leopards than cheetahs indicate that in leopards individuals with higher activity of GPx also tended to have higher activity of SOD. Leopards actually had a higher GPx/SOD ratio (females = 0.55, males = 0.51) than that of cheetahs (females = 0.32, males = 0.49), indicating that leopards had a more balanced ratio between activities of the two enzymes. This result appears to be mainly due to cheetah females having high constitutive SOD but much lower GPx activity than other groups. Thus, cheetah females might have high enzymatic capacity to dismutate the excess of inherent superoxide anion generation, but low capacity of removing peroxides, whose production may be a consequence of the routine or long-term stress associated to exploring the territory containing territorial males. Prior work also found that unbalanced activities of the two enzymes GPx and SOD may be associated with ageing (De Haan et al., 1995), diseases status (Park et al., 2007) or pollution (Jayawardena et al., 2017). We currently do not know whether such differences in the GPx/SOD ratio have functional consequences.

Another potential source of differences between cheetahs and leopards might lie in the immune system. We showed previously that the bacterial killing capacity of cheetahs was twice as high as that of leopards (Heinrich et al., 2016, 2017). Part of the bacterial killing capacity relies on the oxidative burst, i.e. the release of free radicals from leucocytes (Sorci and Faivre, 2009). The action of free radicals is not specifically directed against pathogens, thus they may cause oxidative damage to biomolecules such as lipids or proteins (Sorci and Faivre, 2009).

In conclusion, the results of our work demonstrate that female cheetahs had a different blood oxidative profile as compared to male cheetahs and leopards, possibly reflecting higher dysregulation of the oxidative balance. Although in our study region, female cheetahs do not show any noticeable signs of reduced fitness, our results call for further work to understand whether females either face higher reproductive costs than female leopards or are being exposed to an unknown source of stress. Physiological measurement tools have strong potential to uncover unnoticed costs that might negatively affect fitness in the long-term, particularly in threatened species such as the cheetah and the leopard. Thus, the use of oxidative status markers in combination with other physiological markers might enhance the success of conservation and management programs of endangered carnivores.

Although cheetahs and leopards are phylogenetically related, they showed both similarities and differences in blood oxidative status. This might be based on their different life histories, which would not corroborate the assumption that two such species consistently differ in key physiological traits. A multi-species approach would be needed to infer on physiological adaptations (Garland and Adolph, 1994). Finally, our work showed how the use of multivariate models may help to detect differences in oxidative status that univariate approaches may not detect.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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