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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