

Oxidative stress predicts long-term resight probability and reproductive success in Scopoli's shearwater (*Calonectris diomedea*)

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A major challenge in conservation physiology is to find out biomarkers that reliably reflect individual variation in wear and tear. Recent work has suggested that biomarkers of oxidative stress may provide an additional tool to assess the health state of individuals and to predict fitness perspectives. In this study, we assessed whether three biomarkers of plasma oxidative status predicted the following factors: (i) the resight probability as breeder in the next seasons; and (ii) the cumulative reproductive output over multiple years in Scopoli's shearwaters (*Calonectris diomedea*) using a 7 year individual-based data set. Our results show that shearwaters having higher levels of a marker of oxidative damage (reactive oxygen metabolites) in 2008 had a lower resight probability in the next years and a lower number of chicks raised from 2008 to 2014. In contrast, two biomarkers of antioxidant defences (non-enzymatic antioxidant capacity of plasma and thiols) did not have any predictive value. Increased concentrations of plasma reactive oxygen metabolites, together with the significant individual repeatability over time in this metric of oxidative stress found in numerous studies, suggest that this metric might serve as a blood-derived biomarker for health and fitness perspectives in birds and, possibly, also in other taxa.

Key words: Antioxidant, biomarker, conservation, oxidative damage, seabirds, thiols

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Introduction

Within natural animal populations, individuals can differ dramatically in the degree to which they have accumulated or produced damage as a result of past experiences. One of the major challenges in conservation physiology is to find out biomarkers (measurable indicators of a given biological state) that reliably reflect this individual variation in wear and tear and that may predict individual fitness perspectives (Bennett, 1987; Cooke *et al.*, 2013; Beaulieu and Costantini, 2014; Dantzer *et al.*, 2014). It is increasingly advocated that physiological biomarkers of

stress may provide a valuable tool for long-term environmental monitoring programmes of natural 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).

Measures of physiological stress, such as the hormones cortisol and corticosterone (stress hormones), have become key biomarkers of animal population health in conservation studies because secretion of these hormones is increased in

response to exposure to environmental stressors (Romero, 2004; Busch and Hayward, 2009; Wingfield, 2013; Dantzer *et al.*, 2014). However, stress hormones do not provide a direct currency of genuine damage incurred by the organism. Recent work has suggested that biomarkers of oxidative stress may provide an additional tool to assess the health state of individuals and to predict fitness perspectives, because they provide a more direct quantification of some aspects of tissue damage (Beaulieu and Costantini, 2014; Hau *et al.*, 2015).

Oxidative stress is a complex multifaceted biochemical stressor for cells that manifests as oxidative damage to biomolecules and antioxidant depletion (Sies, 1991; Halliwell and Gutteridge, 2007). Biomarkers of oxidative status are routinely being applied in epidemiological, clinical and veterinary research because increased oxidative stress has been associated with several diseases and cell senescence (Halliwell and Gutteridge, 2007). Moreover, toxicological research has provided evidence that biomarkers of oxidative status are very sensitive to exposure to both organic and non-organic contaminants (Isaksson, 2010). Although it is increasingly recognized that the need to manage oxidative stress in an optimal way may also be an important mechanism driving the outcome of many life-history trade-offs (Costantini, 2008, 2014; Helfenstein *et al.*, 2010; Metcalfe and Alonso-Alvarez, 2010; Travers *et al.*, 2010; Isaksson *et al.*, 2011; Blount *et al.*, 2015), little work has been done so far in conservation research. Studies on wild birds have, for example, found that biomarkers of oxidative damage may predict the probability to return to the breeding ground the next year in the common yellowthroat (*Geothlypis trichas*; Freeman-Gallant *et al.*, 2011) or the probability to recruit into the population in the European shag (*Phalacrocorax aristotelis*; Noguera *et al.*, 2011), but do not predict return rate in the Adélie penguin (*Pygoscelis adeliae*; Beaulieu *et al.*, 2011). Work has also shown that a marker of plasma non-enzymatic antioxidant capacity (OXY assay) predicts long-term survival in a passerine bird (Saino *et al.*, 2011), but it does not predict the return rate to the colony in the Adélie penguin (Beaulieu *et al.*, 2011). Likewise, an assay of *in vitro* red blood cell resistance to oxidation did not predict recruitment in the great tit (*Parus major*; Losdat *et al.*, 2013). Further work in captive mice (*Mus musculus*) or wild starlings (*Sturnus vulgaris*) has also shown that individuals with lower oxidative stress had higher reproductive success (Stier *et al.*, 2012; chapter 7 of Costantini, 2014).

Although this work has fostered rapid progress in oxidative stress ecology, we lack studies that track the survival and reproductive history of free-living individuals over many years and relate these demographic variations to multiple markers of oxidative status. To this end, in the present study of Scopoli's shearwaters (*Calonectris diomedea*), using a 7 year individual-based dataset we assessed whether three biomarkers of plasma oxidative status measured in a given year predicted the following factors: (i) the resight probability (proxy of survival/reproductive perspectives) as a breeder in the next seasons; and (ii) the cumulative reproductive output over multiple years. In shearwaters, both sexes contribute equally to

incubation and chick rearing (Hamer *et al.*, 2002). Therefore, we also tested whether males and females differed in plasma oxidative status and resight probability, predicting no difference because they share the costs of reproduction.

Materials and methods

Study species and study area

Scopoli's shearwater is a pelagic seabird that breeds in the Mediterranean (Massa and Lo Valvo, 1986). This seabird species is currently listed as Least Concern by the International Union for the Conservation of Nature (www.iucnredlist.org); however, the population trend appears to be decreasing (www.iucnredlist.org). Our study population breeds on Linosa (35°52' N, 12°52' E), a volcanic island off Sicily, which holds the second largest colony of shearwaters in the Mediterranean (Massa and Lo Valvo, 1986; Baccetti *et al.*, 2009). The birds breed inside crevices in the lava formation and are mostly concentrated on the coast of Mannarazza, on the northern side of the island, where field work has been carried out since 2007. They lay their single egg from the second half of May onwards, and chicks hatch between mid-July and the first week of August. Fledglings typically leave the colony around the end of October.

Sampling

From 5 to 9 August 2008, we collected blood samples from 18 breeding adult shearwaters (eight females and 10 males; five pairs). A sample of venous blood was taken from the wing vein a few minutes after capture; the blood was centrifuged and the plasma stored at -20°C until laboratory analyses, which occurred within 2 months from collection. Since 2007, the breeding activity of shearwaters breeding on the coast of Mannarazza has been monitored yearly from May to October in order to record the reproductive history of each individual. Each nest is numbered and mapped, and all the breeding birds have been ringed in order to enable individual identification. Our data set includes data on resight probability and reproductive success collected from 2008 and 2014. Scopoli's shearwaters have a long lifespan (at least 25 years according to the European longevity records of Fransson *et al.*, 2010) and reach sexual maturity after 5 years (Thibault *et al.*, 1997). Hence, our study period represents ~35% of shearwater lifespan post-sexual maturity.

Laboratory analyses

Three biomarkers of plasma oxidative status were measured according to established protocols for vertebrates (e.g. Costantini *et al.*, 2011). The reactive oxygen metabolites (d-ROMs) assay (Diacron International, Grosseto, Italy) was used to measure plasma oxidative damage metabolites (mostly organic hydroperoxides) that are generated early in the oxidative cascade. The OXY-Adsorbent test (Diacron International) was used to quantify the ability of non-enzymatic antioxidants present in plasma (e.g. vitamins C, A and E and uric acid) to cope with the *in vitro* oxidant action of hypochlorous

acid (HOCl; an endogenously produced oxidant). The –SHp test (Diacron International) was used to quantify the concentration of total thiols (e.g. albumin, lipoic acid and glutathione) present in plasma. The ROMs were expressed as millimolar of H₂O₂ equivalents; OXY values were expressed as millimolar of HOCl neutralized; and thiols were expressed as micromolar of SH groups.

Statistical analyses

Analyses were run using R (version 3.1.1; R Core Team, 2013). Generalized linear mixed models (glmer in package ‘lme4’) with a binomial error distribution and a logit link function were used to test the effects of sex and of each single biomarker of oxidative status on the individual resight probability. Each bird had an individual resight history, where 0 and 1 indicated whether the bird was not resighted or was resighted in a given year, respectively. Hence, we had a repeated-measures design. For example, if a bird was seen for 2 years consecutively, but not for the next 5 years, it had the following seven repeated measures: 1, 1, 0, 0, 0, 0 and 0. Outcomes were unchanged if the total number of times a bird was resighted at the colony after 2008 was used as a response variable (ROMs, $P = 0.005$; OXY, $P = 0.86$; and thiols, $P = 0.07$). In each model, we entered sex and a biomarker (ROMs, OXY or thiols, respectively) as fixed factors. Nest was entered as a random factor; individual was entered as a random factor nested within nest. Linear mixed models (lmer in package ‘lme4’) were used to test the covariation between number of chicks reared by each individual in the period 2008–2014 and each single biomarker. Linear mixed models were also used to compare each oxidative status metric between birds that were able to return to their nest each year and those that were not. This approach enabled us to eliminate any confounding effect of resight rate on the covariation between oxidative status and reproductive output. In each model, nest was entered as a random factor. Finally, Student’s unpaired t -test was used to compare biomarkers of plasma oxidative status between sexes.

Results

Eight of the 18 shearwaters sampled in 2008 bred successfully each year over the study period. Four shearwaters were not resighted again after the first sampling season of 2008. The other six shearwaters were resighted as breeders from one to five consecutive seasons between 2009 and 2014. When a bird was not resighted in our population at year t , it was not resighted again in the following breeding seasons. Both mates were resighted each year for two of the five pairs included in our data set.

The three biomarkers were not correlated with each other (ROMs vs. OXY, $r = 0.13$, $P = 0.61$; ROMs vs. thiols, $r = 0.39$, $P = 0.11$; and OXY vs. thiols, $r = -0.12$, $P = 0.65$), indicating that they provided different information about the plasma oxidative status. Shearwaters having higher plasma ROMs in 2008 had lower resight probability in the following seasons (Table 1 and Fig. 1); OXY and thiols did not significantly predict the resight

Table 1: Outcomes of generalized linear mixed models that show the effects of sex and of each single biomarker of oxidative status on the individual resight probability

| | Estimate | SEM | P -value |
|--------|---------------|--------------|--------------|
| Sex | −0.646 | 1.243 | 0.603 |
| ROMs | −2.662 | 0.855 | 0.002 |
| Sex | 1.486 | 1.993 | 0.456 |
| OXY | 0.001 | 0.711 | 0.999 |
| Sex | 1.310 | 1.247 | 0.294 |
| Thiols | −1.033 | 0.566 | 0.068 |

Abbreviations: ROMs, reactive oxygen metabolites; OXY, non-enzymatic anti-oxidant capacity of plasma.

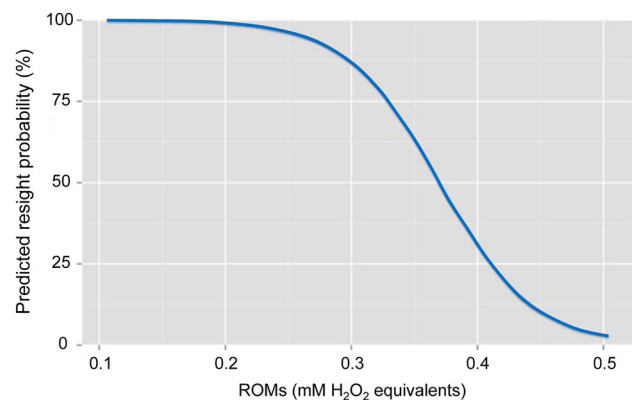


Figure 1: Predicted resight probability of adult shearwaters as function of plasma oxidative damage (ROMs). The fitted line shows the predicted resight probability from generalized linear mixed models.

probability (Table 1). Shearwaters having less ROMs in 2008 successfully reared more offspring over our study period (estimate \pm SEM -1.69 ± 0.45 , $P = 0.0002$). Neither OXY (estimate \pm SEM -0.14 ± 0.63 , $P = 0.082$) nor thiols (estimate \pm SEM -1.03 ± 0.52 , $P = 0.073$) were related to reproductive success (Fig. 2). Consistently, those individuals that were able to return to their nest each year had lower ROMs ($P = 0.001$) but similar OXY and thiols ($P \geq 0.29$; Fig. 3) compared with those that did not return. Males and females had similar values of ROMs (mean \pm SEM 0.32 ± 0.02 and 0.32 ± 0.05 mM H₂O₂ equivalents, respectively), OXY (mean \pm SEM 316.0 ± 9.7 and 320.1 ± 30.7 mM HOCl neutralized, respectively) and thiols (mean \pm SEM 344.4 ± 42.2 and 343.5 ± 45.4 μ M of SH groups, respectively; Student’s unpaired t -test, P -value ≥ 0.90) and resight probability in our study area (Table 1).

Discussion

One of the aims of our work was to identify potential blood-derived biomarkers of fitness-related traits in a seabird using a 7 year individual-based data set. The results of our work show

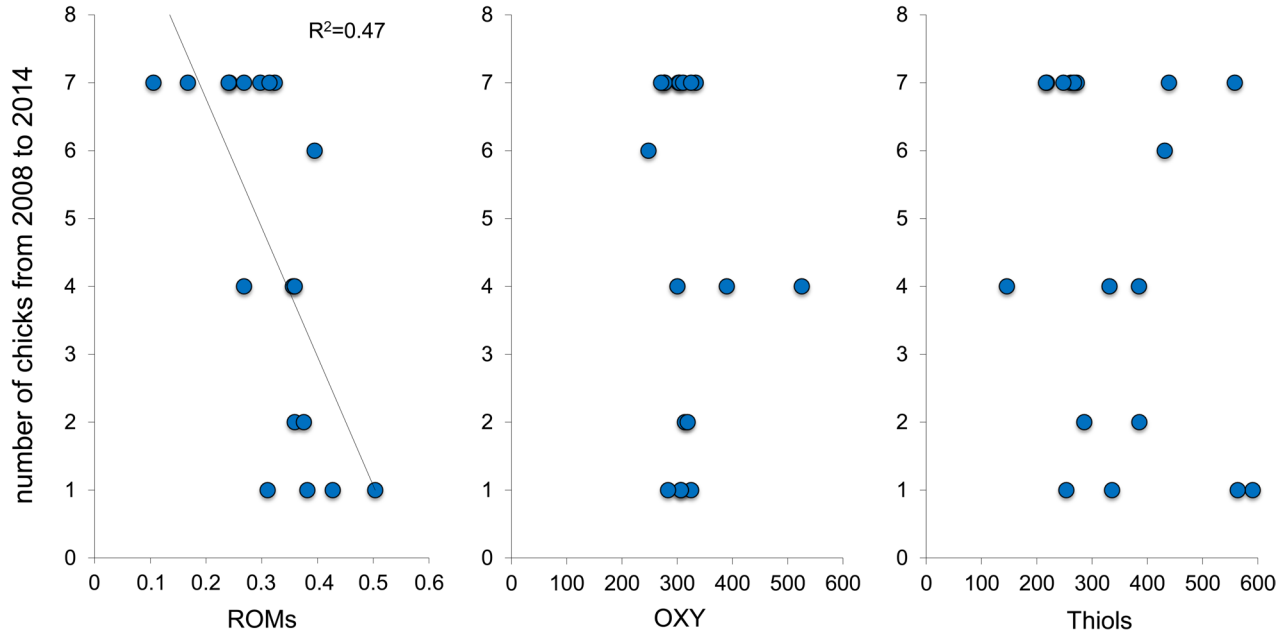


Figure 2: Number of chicks raised successfully by each individual from 2008 to 2014 in relationship to plasma oxidative damage (ROMs, in millimolar of H₂O₂ equivalents), plasma non-enzymatic antioxidant capacity (OXY, in millimolar of HOCl neutralized) and plasma thiols (in micromolar of SH groups). Regression lines are shown when $P \leq 0.05$.

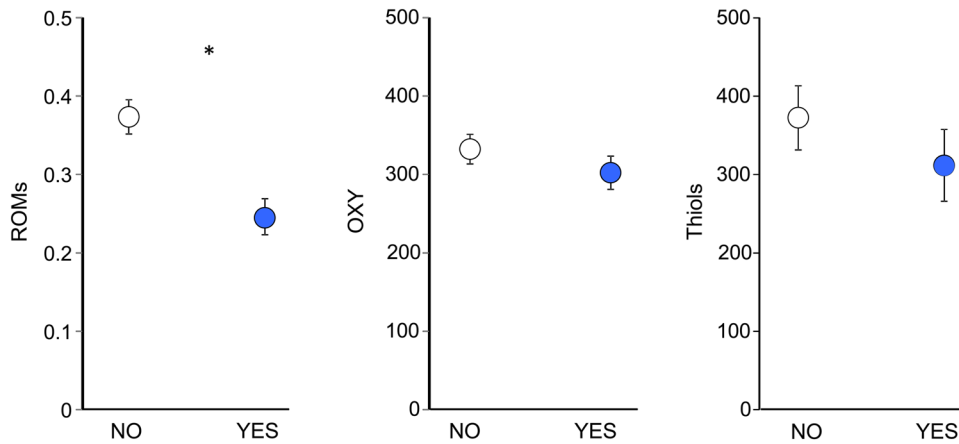


Figure 3: Comparison of metrics of plasma oxidative status between shearwaters that returned to their nest each year (YES) and those that did not (NO). Values are shown as least-squares means \pm SEM. *Significant difference at $P \leq 0.05$. ROMs are expressed as millimolar of H₂O₂ equivalents; OXY values are expressed as millimolar of HOCl neutralized; and thiols are expressed as micromolar of SH groups.

that a marker of plasma oxidative damage (d-ROMs) predicted the resight probability as a breeder and the cumulative reproductive success in Scopoli's shearwater over multiple years. Conversely, two biomarkers of plasma antioxidant defences did not have any predictive power in our species. We do not know whether the birds that were not longer resighted left the study area, died or stopped reproducing. Shearwaters are highly philopatric and use the same nest every breeding season (Ristow *et al.*, 1990; Thibault, 1993; Borg and Cachia-Zammit, 1997; Rabouam *et al.*, 1998). In long-lived seabirds,

intermittent non-breeding years (so-called 'sabbatical years') are common. It is thought that birds skip reproduction (and so take sabbaticals) when they are in poor condition such that the costs of reproduction would be too high to sustain (Giudici *et al.*, 2010; Goutte *et al.*, 2011). Shearwaters do take sabbaticals, but in the next breeding season most of the birds return to the same nest for breeding (Mougin *et al.*, 1997). We therefore consider it likely that the birds that we did not longer observe died or entered a prolonged non-reproductive phase (sabbaticals may last for more than 1 year; Mougin *et al.*, 1997).

The d-ROMs assay measures oxidative damage (mostly organic hydroperoxides generated by oxidation of fatty acids, cholesterol, proteins and nucleic acids) that is generated early in the oxidative cascade. Previous studies showed that this assay is very sensitive to a shift of the cell oxidative balance toward more oxidizing conditions (e.g. exposure to hypoxia/reoxygenation, Bertuglia and Giusti, 2003; exposure to hyperbaric oxygen, Benedetti *et al.*, 2004). High levels of ROMs have been associated with numerous pathological states in humans (e.g. Vassalle *et al.*, 2013; Mori *et al.*, 2014) or dogs (e.g. Winter *et al.*, 2009; Paltrinieri *et al.*, 2010; Finotello *et al.*, 2014). Work on wild vertebrates also showed that ROMs can be increased by immune response (Costantini, 2014), stress hormones (Lucas and French, 2012) or malaria infection (van de Crommenacker *et al.*, 2012). Hence, individual variation in oxidative damage in shearwaters was probably due to the sum of several sources of oxidative stress, including the effect of ageing, as well as exposure to environmental stressors, such as parasites or food shortage. Hence, our marker of damage was capable of reflecting individual variation in wear and tear that impinged on our proxy for survival/reproductive perspectives (i.e. resight probability). Increased concentrations of plasma ROMs consequent to stressor exposure, together with the significant intra-individual repeatability over time in this metric of oxidative stress found in numerous studies (e.g. Costantini *et al.*, 2007; Hau *et al.*, 2015), suggest that this metric might serve as a biomarker for health and fitness perspectives in birds and, possibly, also in other taxa.

Selection of biomarkers is not straightforward, however, because biomarkers may provide different information about the individual condition and their diagnostic value might differ among species. Although in our study, the OXY biomarker was not related to resight probability or to reproductive success, studies on other species have found different results. Using longitudinal data, Saino *et al.* (2011) showed that high levels of the OXY biomarker positively predicted long-term survival in wild barn swallows (*Hirundo rustica*) while controlling for possible confounding effects of individual age. The effect of OXY on viability was relatively strong, because an increase of one standard deviation in antioxidant capacity was positively associated to a ~20% change in annual survival (Saino *et al.*, 2011). Using the same OXY assay as that used for barn swallows, Beaulieu *et al.* (2013) found that Gentoo (*Pygoscelis papua*) and Adélie penguins (*P. adeliae*) from growing populations had higher OXY than penguins from decreasing populations, and this was true when considering each penguin colony independently or irrespective of species, location and levels of plasma oxidative damage. Other studies that used the OXY assay, however, did not find any associations between this biomarker and survival (e.g. Beaulieu *et al.*, 2011; van de Crommenacker, 2011). These results suggest that there is variation among species in the biological information provided by a same biomarker. This raises the need for the following: (i) use of multiple biomarkers in order to obtain more information about oxidative status of the individual; and (ii) examination of how the biomarkers of oxidative status relate to environmental stressors and fitness components. These two

approaches would enable conservation practitioners to confirm that a given biomarker of oxidative status provides information that is ecologically relevant and may therefore be useful in conservation studies (Beaulieu and Costantini, 2014).

Although work on laboratory rodents showed that reduced concentrations of intracellular thiols are indicative of physiological dysregulation and loss of cell function (Jones, 2006; Sohal and Orr, 2012), our metric of thiol concentration tended to be higher (but not significantly) in birds with lower resight probability and was not related to reproductive success. A reason for this might lie with our measurement of thiol concentration being carried out on plasma. Although decreased circulating thiols have been associated with human pathological states (Halliwell and Gutteridge, 2007) and, in birds, with environmental stress (Isaksson *et al.*, 2005; Isaksson, 2010) or with a larger investment in reproduction (Costantini, 2010; Costantini *et al.*, 2010), it might be that the concentrations of circulating thiols are so high as to make them capable of buffering moderate amounts of oxidative stress, meaning that significant changes in plasma thiols may be detected only when oxidative stress is severe. This might also suggest that plasma thiols have high resilience, i.e. high capability of recovering from moderate stressors. There was, however, a tendency for thiols to be higher in birds with lower resight probability. This might indicate that these birds needed to upregulate this group of antioxidants slightly to withstand increased production of ROMs. Thiols are used as cofactors of glutathione peroxidase, an antioxidant enzyme that reduces ROMs to their corresponding alcohols (Halliwell and Gutteridge, 2007). This scenario is supported to some extent by a moderate, though not significant, positive correlation ($r = 0.39$) between ROMs and thiols. Further work is needed to clarify the usefulness of plasma thiols as a biomarker of health and the way in which they change in response to exposure to environmental stressors.

There is now much interest in the role of oxidative stress as a cost and constraint of reproduction (Costantini, 2008, 2014; Helfenstein *et al.*, 2010; Metcalfe and Alonso-Alvarez, 2010; Travers *et al.*, 2010; Isaksson *et al.*, 2011; Blount *et al.*, 2015). Our data give support to the hypothesis that oxidative stress might constrain future reproductive success. Whether a high oxidative stress level directly causes a decrease in reproductive or survival perspectives is, however, unclear because of the correlative nature of our data.

Our work also shows that males and females did not differ in plasma oxidative status while chick rearing. In shearwaters, both sexes contribute equally to incubation and chick rearing (Hamer *et al.*, 2002), which might explain why we did not observe any differences between them. In line with this finding, the sexes also did not differ in their resight probability.

We did not find any significant correlations among our three physiological metrics, indicating that they did not provide redundant information about the plasma oxidative status. A review by Dotan *et al.* (2004) revealed that metrics of oxidative stress correlated with each other only in severe pathological

conditions. In other circumstances, Dotan *et al.* (2004) suggested that different types of oxidative stress may prevail over others. This highlights the importance of the following: (i) collecting multiple metrics of oxidative stress; (ii) recognizing that increased oxidative damage to a certain group of biomolecules does not necessarily result in increased damage to other biomolecules; and (iii) testing the fitness consequences of different kinds of damage and antioxidant defences.

In conclusion, our study showed that, in the context of the emerging field of conservation physiology, selected blood-based biomarkers of oxidative stress might represent valuable metrics to predict individual fitness perspectives.

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