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RESEARCH ARTICLE

Inflammatory challenge increases measures of oxidative stress in a free-ranging, long-lived mammal

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SUMMARY

Oxidative stress – the imbalance between reactive oxygen species (ROS) and neutralising antioxidants – has been under debate as the main cause of ageing in aerobial organisms. The level of ROS should increase during infection as part of the activation of an immune response, leading to oxidative damage to proteins, lipids and DNA. Yet, it is unknown how long-lived organisms, especially mammals, cope with oxidative stress. Bats are known to carry a variety of zoonotic pathogens and at the same time are, despite their high mass-specific basal metabolic rate, unusually long lived, which may be partly the result of low oxidative damage of organs. Here, we asked whether an immune challenge causes oxidative stress in free-ranging bats, measuring two oxidative stress markers. We injected 20 short-tailed fruit bats (*Carollia perspicillata*) with bacterially derived lipopolysaccharide (LPS) and 20 individuals with phosphate-buffered saline solution (PBS) as a control. Individuals injected with LPS showed an immune reaction by increased white blood cell count after 24 h, whereas there was no significant change in leukocyte count in control animals. The biological antioxidant potential (BAP) remained the same in both groups, but reactive oxygen metabolites (ROMs) increased after treatment with LPS, indicating a significant increase in oxidative stress in animals when mounting an immune reaction toward the inflammatory challenge. Control individuals did not show a change in oxidative stress markers. We conclude that in a long-lived mammal, even high concentrations of antioxidants do not immediately neutralise free radicals produced during a cellular immune response. Thus, fighting an infection may lead to oxidative stress in bats.

Key words: reactive oxygen metabolites, immune response, bat, antioxidants.

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INTRODUCTION

All aerobic organisms produce reactive oxygen species (ROS) as by-products during energy production in mitochondria. In fact, it is estimated that around 2–3% of oxygen consumed by cells is diverted to generate superoxide and hydrogen peroxide, two of the most important ROS (Chance et al., 1979). ROS are highly reactive and thus can lead to oxidative damage by peroxidation of membrane fatty acid chains, modification of DNA and loss of sulfhydryls and carbonylation in proteins (Sohal et al., 1990; Sohal and Weindruch, 1996; Goyns, 2002). An animal can mitigate these negative effects by raising an antioxidant barrier, which may consist of both exogenous, diet-derived antioxidants such as vitamin E, and endogenously produced antioxidants such as uric acid or antioxidant enzymes (e.g. superoxide dismutases and peroxidases), converting ROS into less reactive molecules. An imbalance between pro-oxidants and antioxidants results in oxidative stress, which may impair the metabolism of an organism by causing oxidative damage as described above (Rose et al., 2002). Oxidative stress causes senescence in cells and is therefore hypothesised to be an important modulator of life-history trade-offs in vertebrates (Costantini, 2008; Nussey et al., 2009); it has consequently been regarded as the main cause of ageing in the literature of past decades (Harman, 1955). However, the free-radical theory of ageing has recently been challenged, as experimental and correlative studies do not always support the hypothesis that high oxidative stress leads to shorter lifespans (Speakman and Selman, 2011).

As the creation of ROS generally increases proportionately with the amount of energy produced, i.e. with mass-specific metabolic rate, animals with a high mass-specific metabolic rate should have a shorter life span than species with a low mass-specific metabolic rate (Pearl, 1928; Harman, 1955; Sacher, 1959). Evidence for this free-radical theory of ageing suggested by Harman (Harman, 1955) has been found in many empirical studies. In general, small mammals with relatively high basal metabolic rates have lower life expectancies than large mammals with low basal metabolic rates (Hulbert et al., 2007). Variations in longevity among species have also been shown to correlate negatively with the amount of superoxide anion radicals produced in mitochondria (Tolmasoff et al., 1980; Sohal and Weindruch, 1996) and oxidative damage of mitochondrial DNA (Adelman et al., 1988; Barja and Herrero, 2000).

The negative correlation of mass-specific metabolic rate and longevity among mammals comes with a few exceptions: for example, small-sized bats may live about 3–4 times longer than similar-sized terrestrial mammals (Austad and Fischer, 1991; Wilkinson and South, 2002), but at the same time, their metabolic rates are exceptionally high because of their ability of powered flight (Munshi-South and Wilkinson, 2010). Thus, the question arises of whether the high mass-specific metabolic rate of bats produces more pro-oxidants than that of similar-sized terrestrial mammals, and if so, whether bats show increased oxidative damage. If bats indeed have to cope with high oxidative stress, what factor predisposes them for long life expectancies? Initial studies on oxidative stress

in bats have shown that bats have lower levels of protein oxidation than terrestrial mammals (Brunet-Rossinni, 2004). Potentially, bats may have potent repair mechanisms for damage caused by oxidation. It has recently been shown that genes regulating repair mechanisms for DNA damage are positively selected for in bacteria (Sghaier et al., 2008) and potentially also in vertebrates. However, low oxidative damage in bats may also be explained by (1) low pro-oxidant production, (2) high antioxidant levels, or (3) a combination of both. Indeed, bats seem to produce lower levels of pro-oxidants than terrestrial mammals (Brunet-Rossinni, 2004), and also have higher levels of both enzymatic and non-enzymatic antioxidants in their organs (Wilhelm Filho et al., 2007). Thus, low oxidative stress may be causative for the exceptional longevity of bats. However, it remains to be investigated why bats have such a low level of oxidative stress and what factors influence the production of pro-oxidants and antioxidants.

Besides their unusually long lifespan, bats are also outstanding with respect to their pathogen load, particularly as a reservoir for important zoonotic pathogens (Wibbelt et al., 2010; Wood et al., 2012) such as lyssaviruses (Kuzmin et al., 2011), coronaviruses (Li et al., 2005) and paramyxoviruses (Drexler et al., 2012). Although it is crucial to understand how the immune system of bats works and how they defend themselves against these pathogens, surprisingly little is known about bat immunity and the factors influencing it (Dobson, 2005). Recent studies have shown a correlation between immune parameters and ecological factors such as dietary niche and roost use in bats (Allen et al., 2009; Schneeberger et al., 2013). Also, experiments on Mexican free-tailed bats (*Tadarida brasiliensis*) demonstrated that they can mount a considerable cellular immune response after injection of mitogens such as phytohaemagglutinin (Allen et al., 2009). As in most other animals, variations in immune responses can be linked to disease susceptibility, such as the white-nose syndrome in temperate-zone bats that eradicated millions of bats in North America during the last decade (Lorch et al., 2011; Moore, 2011). However, mounting an immune response is not only energetically costly (Lochmiller and Deerenberg, 2000) but also associated with an increased production of ROS. During an immune response, the host metabolic rate is usually elevated (Sheldon and Verhulst, 1996), which leads to higher mitochondrial activity and consequently to increased ROS production (Finkel and Holbrook, 2000). Additionally, different white blood cell subtypes involved in immune responses produce ROS to directly kill pathogens (Dröge, 2002), and to enhance the activation of T-lymphocytes (Dröge, 2002; Reth, 2002). Thus, ROS have a signalling function during an immune response and a direct negative effect on parasites and pathogens, but can at the same time also damage the tissue of the host. Mounting an immune response therefore should not only lead to an increase of ROS but also change antioxidant levels to mitigate the negative effect. A meta-analysis of avian studies has found a positive association between immune responses and oxidative stress markers; however, findings on how immune responses influence both pro-oxidants and antioxidants are inconsistent (Costantini and Møller, 2009). Furthermore, some of these studies only involve either pro-oxidants or antioxidants, yet it is important to measure both in order to assess the level of oxidative stress (Costantini and Verhulst, 2009). Also, carotenoids, which are among the most frequently assessed antioxidants in birds, have recently been shown to play a rather minor role in the antioxidant defence of birds (Costantini and Møller, 2008).

As bats are special with respect to their longevity, high mass-specific metabolic rate and disease susceptibility, but apparently show low oxidative damage, our aim was to study whether an immune response leads to an increase in oxidative stress in bats. Most studies on birds show an increase in ROS and a decrease in antioxidants after an immune challenge (Costantini and Møller, 2009). Because birds and bats have high metabolic rates, we would expect a similar effect of immune activation on oxidative stress for the two taxa. However, antioxidants used to counterbalance pro-oxidants may differ between birds and bats: two essential antioxidants, α-tocopherol and retinol, have been found in all Neotropical bat species investigated so far (Müller et al., 2007), while β-carotene and lutein, among the most important antioxidants in birds, were missing in five out of six bat species. Furthermore, in contrast to many birds (Chaudhuri and Chatterjee, 1969), bats – just like haplorhine primates, including humans (*Homo sapiens*), capybaras (*Hydrochoerus hydrochaeris*) and guinea pigs (*Cavia porcellus*) – are unable to synthesise vitamin C because they lack L-gulonolactone oxidase (Birney et al., 1976). Thus, it might not be feasible to extrapolate findings from birds to bats and other mammals.

Here, we conducted an immune challenge experiment in the short-tailed fruit bat, *Carollia perspicillata* (Linnaeus 1758), an abundant, frugivorous bat species commonly found in lowland regions of the Neotropics. This species can be kept in captivity for short periods and has been used before in immunological studies (Greiner et al., 2010). We asked whether mimicking a bacterial infection *via* injection of lipopolysaccharide (LPS) and the resulting immune response leads to a change in reactive oxygen metabolites (ROMs), representing total ROS produced, and antioxidant level. Our expectation was that the concentration of ROMs would increase and the level of antioxidants would decrease in the bats in order to avoid oxidative stress.

MATERIALS AND METHODS

We captured 12 *C. perspicillata* (six males and six females) in November and December 2011 and 28 *C. perspicillata* (14 males and 14 females) in November and December 2012, respectively, within the vicinity of 'La Selva' Biological Station (10°25′N, 84°00′W, Province Heredia, Costa Rica) using nylon mist nets (2.5 m height; Ecotone, Gdynia, Poland) at ground level. The experiments took place within 1 week of capture of the bats. We marked bats individually and kept them in an outdoor flight cage (3.4×6.1×2.5 m) where they were fed *ad libitum* with banana and papaya and provided with water. The experiment started after all bats had been allowed to habituate to the captive conditions for at least 3 days.

At the start of the experiment, we caught all animals in the flight cage, put them in individual cotton bags and weighed them using a spring balance (50 g capacity, Pesola, Baar, Switzerland). We took an initial blood sample of ~60 µl from each individual by puncturing the antibrachial vein with a sterile needle (no. C721.1, Carl Roth GmbH, Karlsruhe, Germany) and transferring blood drops into heparinised capillary tubes (no. 521-9100, VWR, Darmstadt, Germany). In these samples, we measured basal antioxidant status and immunity (see below). Then we assigned half of the individuals of each sex randomly to the experimental group and the other half to the control group. Animals from the experimental group were injected subcutaneously with 50 µl of 1 mg ml^{-1} LPS (*Escherichia coli*, no. L2630, Sigma-Aldrich, Munich, Germany) in phosphate-buffered saline solution (PBS; no. L1825, Biochrom AG, Berlin, Germany) using a sterile disposable syringe (no. 0053.1, Carl Roth GmbH). LPS is an endotoxin that induces an immune reaction in treated animals, as well as sickness behaviour such as reduced locomotion and feeding behaviour (Kozak et al., 1994). Furthermore, endotoxins are known to generally increase oxidative stress markers

(Victor et al., 2004), leading to oxidative damage (Skibska et al., 2006). Individuals of the control group were injected with 50 µl PBS without the antigen. We weighed each bat again 24 h post-injection and took an additional blood sample. Because of the small sample volume, we were restricted to measuring only part of the likely complex immunological response to LPS. We therefore produced blood smears and performed total white blood cell (WBC) counts as a proxy for detecting an immunological reaction to LPS. The remaining blood samples were centrifuged and the plasma was taken and stored at −80°C until further analysis of oxidative stress parameters. All bats were released at their site of capture after collection of the final blood sample.

Blood smears were stained with May–Gruenwald's solution (no. T863.2, Carl Roth GmbH) and Giemsa (no. T862.1, Carl Roth GmbH). We manually estimated total WBC count by counting the cells in 10 visual fields with a microscope under 200× magnifications (Schneeberger et al., 2013).

We measured markers of oxidative stress [dROM and biological antioxidant potential (BAP)] using the Free Radical Analytical System (FRAS4 evolvo; H&D srl, Parma, Italy), following the instructions provided by the manufacturer. We measured the concentration of ROMs using dROM-kits, which represents the total level of hydroperoxide in plasma that is created during peroxidation of amino acids, lipids and proteins, representing free radicals from which ROMs are formed (Alberti et al., 2000; Buonocore et al., 2000). We added 10 µl of plasma to a buffered chromogen, where the derivates of ROMs form a coloured compound that can be measured photometrically at a maximum absorbency peak of 505 nm after 5 min of incubation at 37°C. According to Lambert–Beer's law, the absorbance is directly proportional to the concentration of ROMs and is expressed as U Carr, where 1 U Carr is equivalent to 0.08 mg dl^{-1} hydrogen peroxide. The antioxidant potential of the plasma was measured using BAP-kits. We dissolved 10 µl of plasma into a coloured solution containing ferric ions ($FeCl_3$) and a chromogenic substrate (a sulphur-derived compound). After 5 min of incubation at 37°C, we measured the degree of decolouration by the plasma antioxidants by photometry with FRAS4 evolvo. The intensity of decolouration is directly proportional to the ability of the plasma to reduce ferric ions and thus to the concentration of non-enzymatic antioxidants expressed as mmol l^{-1}.

All statistical tests were run using R statistical software (R Development Core Team, 2010). As individuals were not caught and handled at the same time, we tested whether the delay between capture and handling had an effect on measurements of cellular immune response and oxidative stress. We did not find such an influence on WBC count (Spearman rank correlation; ρ=–0.160; P=0.157), dROM (ρ=0.046; P=0.684) or BAP (ρ=0.067; P=0.559), excluding the potential of capture and handling stress to confound our subsequent analysis.

To test whether potential changes in measures of oxidative stress and WBC count could be accounted for by treatment with LPS or PBS, we calculated mixed effects models using the package 'lme4' (Bates et al., 2011) with the interaction between treatment and time of sampling (before or after injection) as well as sex as fixed factors, and of dROM, BAP or WBC count as response variable. We log-transformed the response variables in order to achieve normal distribution of model residuals. Individual identity was included as a random factor in all models to account for repeated measures of the same individual. *P*-values were extracted using the 'pvals.fnc' function of the package 'languageR' (Baayen, 2011). To test whether WBC count correlated with measures of oxidative stress, we conducted Spearman rank correlation tests.

RESULTS

Body mass was significantly connected with treatment and day of sampling (χ^2=12.56; P=0.006), but not with sex (χ^2=0.22; P=0.639). Body mass decreased 24 h after LPS injection (t=–3.77; P<0.001; Fig. 1A), but not after PBS injection (t=–0.51; P=0.612; Fig. 1B). WBC count differed significantly between day of sampling and treatment (χ^2=11.34; P=0.010), but not between males and females (χ^2=1.11; P=0.737). WBC count increased significantly 24 h after injection with LPS (t=2.99; P=0.006; Fig. 1C), but only tended to increase after PBS injection (t=1.84; P=0.070; Fig. 1D).

Baseline dROM for *C. perspicillata* (N=40) averaged 100.33±29.75 U Carr (mean ± 1 s.d.) and BAP was 2294±0.48 µmol l^{-1} before treatment. dROM was significantly linked to treatment and day of sampling (χ^2=12.52; P=0.006), but not to sex (χ^2=0.22; P=0.637). dROM increased significantly after injection with LPS (t=3.34; P=0.001; Fig. 2A), but not after injection with PBS (t=–1.13; P=0.261; Fig. 2B). BAP was not related to day of sampling and treatment (χ^2=3.38; P=0.336; Fig. 2C,D) or to sex (χ^2<0.001; P>0.999).

dROM was in general positively correlated to WBC count (ρ=0.23; P=0.044), but BAP and WBC count were not significantly connected (ρ=0.02; P=0.848).

DISCUSSION

Bats are exceptionally long-lived mammals (Austad and Fischer, 1991; Wilkinson and South, 2002) even though they have higher mass-specific metabolic rates than similar-sized, terrestrial mammals

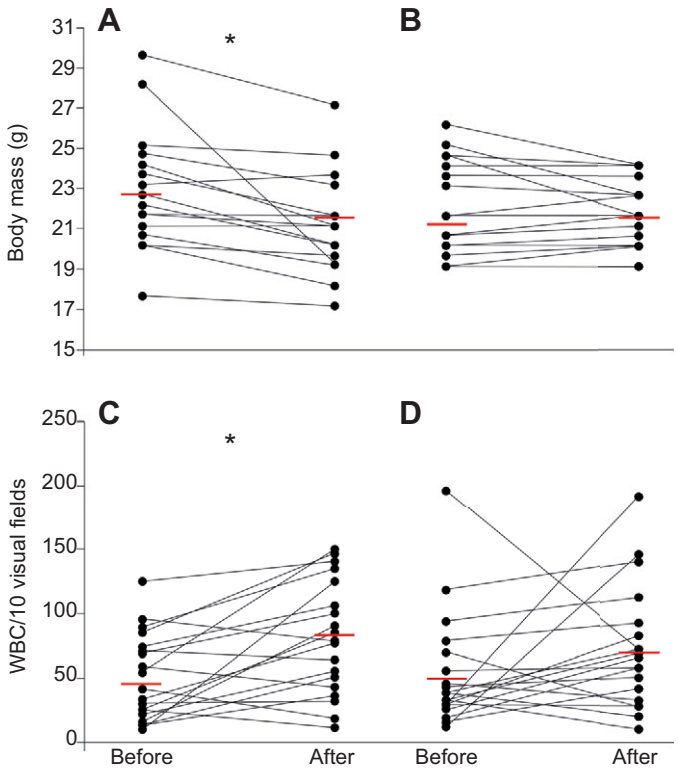

Fig. 1. Body mass decreases in individuals of the experimental group 24 h post-treatment with lipopolysaccaride (LPS; A), but not in individuals of the control group injected with phosphate-buffered saline solution (PBS; B). White blood cell (WBC) count per 10 visual fields increases in animals of the experimental group (C), but not in individuals of the control group (D). Red lines indicate mean values before and after treatment and asterisks indicate a significant difference at P<0.05.

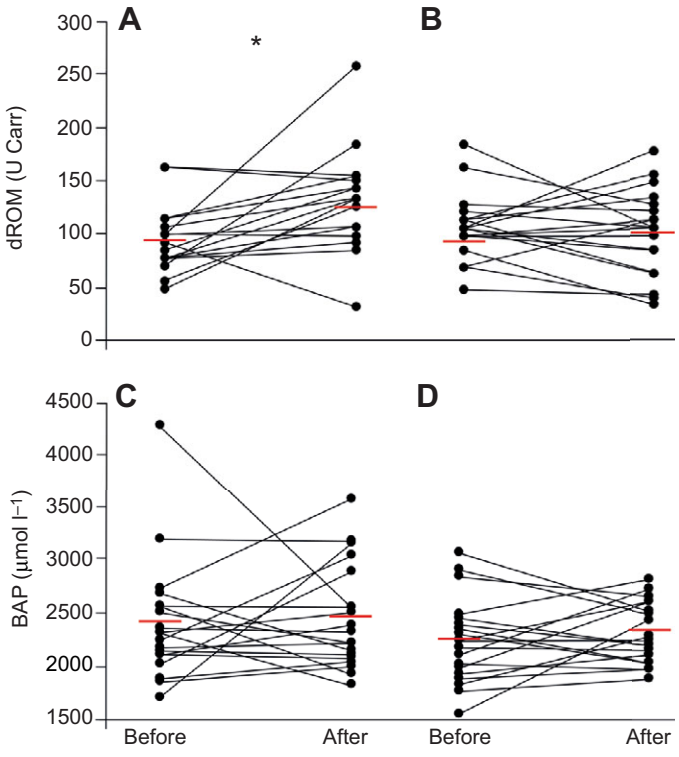

Fig. 2. The concentration of reactive oxygen metabolites (dROM) increases in bats 24 h after injection with LPS (A), but not with PBS as a control (B). Antioxidant concentration (BAP) remained the same in both the experimental (C) and control group (D). Red lines indicate mean values before and after treatment and asterisks indicate a significant difference at $P<0.05$.

(Munshi-South and Wilkinson, 2010). Bats have a lower production rate of ROS (Brunet-Rossinni, 2004) and also lower levels of oxidative damage (Brunet-Rossinni, 2004). Here, we asked whether mounting an immune response in bats leads to an increase in ROMs, representing the total ROS created, and whether this changes the concentration of antioxidants to mitigate oxidative stress. To the best of our knowledge, this is the first study on the effect of mounting an immune response on oxidative stress markers in a free-ranging mammal.

Total WBC count increased significantly in the experimental group 24 h after antigen treatment, but not in the control group. The contrasting results between the control and experimental group indicate that LPS caused a cellular immune reaction in individuals of the experimental group. This is also supported by the observation that bats of the experimental group lost body mass, while body mass remained constant in individuals of the control group. The loss of body mass and associated decrease in food ingestion might be the result of LPS-induced sickness behaviour. In mice, it has previously been shown that LPS results in reduced locomotion and decreased food intake (Kozak et al., 1994).

dROM increased significantly in bats injected with LPS, and WBC count correlated with dROM. Thus, the mounting of a cellular immune response may lead to a higher production of ROS in free-ranging bats, which is then represented by an increased concentration of ROMs in plasma. This pattern is similar to what has been found in various studies on birds (reviewed by Costantini and Møller, 2009). The high level of ROS during a natural infection is usually a combination of oxidants released by the pathogen itself (Halliwell et al., 1993) and the ROS released by the host during the mounting of an immune response (Dröge, 2002; Reth, 2002). As we did not inject bats with active pathogens but with the endotoxin only, we can rule out the possibility of the pro-oxidants being synthesised by the pathogen. Thus, the elevated dROM level in plasma of animals injected with LPS is a consequence of physiological processes involved in sickness behaviour, such as elevated metabolic rate (Finkel and Holbrook, 2000) as well as ROS produced to enhance the cellular immune response and to directly kill pathogens (Dröge, 2002; Reth, 2002). This is supported by the finding that dROM correlated positively with WBC count. Indeed, studies on purified human monocytes showed that LPS directly stimulates the production of superoxide, one of the most important pro-oxidants in vertebrates (Landmann et al., 1995).

The negative effects of ROS on the organism can be mitigated by the production or ingestion of antioxidants. Thus, with an increase of ROS concentration represented by dROM levels in plasma, we would also expect a change in antioxidant levels. However, in our experiment, the concentration of antioxidants remained the same before and after treatment in individuals injected with LPS or PBS. Similar experiments in birds on how antioxidant levels change after mounting an immune response are inconsistent. For example, carotenoid levels but not total non-enzymatic antioxidant levels increased after an immune challenge in wild kestrel nestlings [*Falco tinnunculus* (Costantini and Dell'Omo, 2006)]. In red-legged partridges (*Alectoris rufa*), the total antioxidant concentration remained the same before and after injection with phytohaemagglutinin (Perez-Rodriguez et al., 2008), but increased in greenfinches [*Carduelis chloris* (Hõrak et al., 2007)]. The same was true for chicken (*Gallus gallus domesticus*) injected with LPS (Cohen et al., 2007): there was no change in the level of various antioxidants before and 24 h after treatment. Also, an experimental study on the effect of supplemental feeding of mice with antioxidant-rich wine showed no effect of LPS on total antioxidant levels after 24 h (Percival and Sims, 2000). Similar to the findings of these studies, we found that the antioxidant concentration did not change after treatment with LPS in *C. perspicillata*. Potentially, an antioxidant barrier needs longer than 24 h to be raised, as there may be a time lag between an increase of free radicals and the corresponding antioxidant response (Hõrak and Cohen, 2010; Meitern et al., 2013). In their study on red-legged partridges, Perez-Rodriguez and his colleagues (Perez-Rodriguez et al., 2008) argued that, alternatively, pro-oxidants could have been buffered by carotenoids, which they have measured separately and found to decrease after the immune challenge. However, recently it has been argued that although highly promoted, carotenoids have a rather weak contribution to the avian antioxidant capacity (Costantini and Møller, 2008). Also, carotenoids are not part of the antioxidant barrier in most bats (Müller et al., 2007), which is why the absence of an effect on antioxidants may have been caused by factors other than an immediate buffering of ROS by carotenoids as suggested in birds (Perez-Rodriguez et al., 2008). Potentially, the elevated concentration of ROS may be partly compensated for by a short-term increase of antioxidants ingested by food such as vitamin E or by mobilising enzymatic antioxidants. The study of Percival and Sims (Percival and Sims, 2000) implies that the high level of antioxidants caused by supplemental feeding may not further increase to cope with released ROS when the immune challenge is short. However, as dROM increased after injection with LPS, ROS were apparently not immediately neutralised by antioxidants. Furthermore, the mean baseline BAP of 2294 µmol l^{-1} in *C. perspicillata* indicates a rather moderate concentration of

antioxidants, comparable with the level observed in rats [1874 µmol l^{-1} (Iwata et al., 2010)] and mice [2896 µmol l^{-1} (Maruoka and Fujii, 2012)].

We conclude that an immune response can upregulate some markers frequently associated with oxidative stress even in a long-lived mammal that is known to have a reduced pro-oxidant production compared with terrestrial mammals (Brunet-Rossinni, 2004; Wilhelm Filho et al., 2007). Thus, bats may suffer from long-term consequences of elevated oxidative stress after episodes of acute infection or during chronic infection. The long lifespan of bats is therefore even more puzzling, as bats carry a large variety of pathogens and our results indicate that infections do increase oxidative stress. It remains to be investigated whether natural infections with bat-borne and bat-specific pathogens increase oxidative stress, and how bats cope with the oxidative damage caused by ROS.

LIST OF ABBREVIATIONS

BAP	biological antioxidant potential (mmol l^{-1})
FRAS	free-radical analytic system
LPS	lipopolysaccharide
ROM	reactive oxygen metabolite (concentration given in U Carr)
ROS	reactive oxygen species
WBC	white blood cell

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AUTHOR CONTRIBUTIONS

K.S. and C.C.V. conducted the fieldwork; K.S. counted the white blood cells, measured pro-oxidants and antioxidants, and analysed the data; C.C.V. supervised the study. All authors participated in the design of the study, discussed the results and wrote the manuscript.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

Adelman, R., Saul, R. L. and Ames, B. N. (1988). Oxidative damage to DNA: relation to species metabolic rate and life span. *Proc. Natl. Acad. Sci. USA* **85**, 2706-2708.

Alberti, A., Bolognini, L., Carratelli, M., Della Bona, M. A. and Macciantelli, D. (2000). The radical cation of N,N-diethyl-para-phenylendiamine: a possible indicator of oxidative stress in biological samples. *Res. Chem. Intermediat.* **26**, 253-267.

Allen, L. C., Turmelle, A. S., Mendonça, M. T., Navara, K. J., Kunz, T. H. and McCracken, G. F. (2009). Roosting ecology and variation in adaptive and innate immune system function in the Brazilian free-tailed bat (*Tadarida brasiliensis*). *J. Comp. Physiol. B* **179**, 315-323.

Austad, S. N. and Fischer, K. E. (1991). Mammalian aging, metabolism, and ecology: evidence from the bats and marsupials. *J. Gerontol.* **46**, B47-B53.

Baayen, R. H. (2011). languageR: Data sets and functions with 'Analyzing Linguistic Data: A practical introduction to statistics'. R package version 1.4. http://CRAN.R-project.org/package=languageR

Barja, G. and Herrero, A. (2000). Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. *FASEB J.* **14**, 312-318.

Bates, D., Maechler, M. and Bolker, B. (2011). lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-39. http://CRAN.R-project.org/package=lme4

Birney, E. C., Jenness, R. and Ayaz, K. M. (1976). Inability of bats to synthesise L-ascorbic acid. *Nature* **260**, 626-628.

Brunet-Rossinni, A. K. (2004). Reduced free-radical production and extreme longevity in the little brown bat (*Myotis lucifugus*) versus two non-flying mammals. *Mech. Ageing Dev.* **125**, 11-20.

Buonocore, G., Perrone, S., Longini, M., Terzuoli, L. and Bracci, R. (2000). Total hydroperoxide and advanced oxidation protein products in preterm hypoxic babies. *Pediatr. Res.* **47**, 221-224.

Chance, B., Sies, H. and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59**, 527-605.

Chaudhuri, C. R. and Chatterjee, I. B. (1969). L-Ascorbic acid synthesis in birds: phylogenetic trend. *Science* **164**, 435-436.

Cohen, A., Klasing, K. and Ricklefs, R. (2007). Measuring circulating antioxidants in wild birds. *Comp. Biochem. Physiol.* **147B**, 110-121.

Costantini, D. (2008). Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol. Lett.* **11**, 1238-1251.

Costantini, D. and Dell'Omo, G. (2006). Effects of T-cell-mediated immune response on avian oxidative stress. *Comp. Biochem. Physiol.* **145A**, 137-142.

Costantini, D. and Møller, A. P. (2008). Carotenoids are minor antioxidants for birds. *Funct. Ecol.* **22**, 367-370.

Costantini, D. and Møller, A. P. (2009). Does immune response cause oxidative stress in birds? A meta-analysis. *Comp. Biochem. Physiol.* **153A**, 339-344.

Costantini, D. and Verhulst, S. (2009). Does high antioxidant capacity indicate low oxidative stress? *Funct. Ecol.* **23**, 506-509.

Dobson, A. P. (2005). Virology. What links bats to emerging infectious diseases? *Science* **310**, 628-629.

Drexler, J. F., Corman, V. M., Müller, M. A., Maganga, G. D., Vallo, P., Binger, T., Gloza-Rausch, F., Rasche, A., Yordanov, S., Seebens, A. et al. (2012). Bats host major mammalian paramyxoviruses. *Nat. Commun.* **3**, 796.

Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiol. Rev.* **82**, 47-95.

Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.

Goyns, M. H. (2002). Genes, telomeres and mammalian ageing. *Mech. Ageing Dev.* **123**, 791-799.

Greiner, S., Stefanski, V., Dehnhard, M. and Voigt, C. C. (2010). Plasma testosterone levels decrease after activation of skin immune system in a free-ranging mammal. *Gen. Comp. Endocrinol.* **168**, 466-473.

Halliwell, B., Bomford, A. B., Stern, A., Golenser, J., Chevion, M., Callahan, H. L., Aldunate, J., Morello, A. and Cross, C. E. Balasubramanian et al. (1993). *Free Radicals in Tropical Diseases.* Chur, Switzerland: Harwood Academic Publishers.

Harman, D. (1955). *Aging: A Theory Based on Free Radical and Radiation Chemistry*. Berkeley, CA: University of California Radiation Laboratory.

Hõrak, P. and Cohen, A. (2010). How to measure oxidative stress in an ecological context: methodological and statistical issues. *Funct. Ecol.* **24**, 960-970.

Hõrak, P., Saks, L., Zilmer, M., Karu, U. and Zilmer, K. (2007). Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. *Am. Nat.* **170**, 625-635.

Hulbert, A. J., Pamplona, R., Buffenstein, R. and Buttemer, W. A. (2007). Life and death: metabolic rate, membrane composition, and life span of animals. *Physiol. Rev.* **87**, 1175-1213.

Iwata, N., Okazaki, M., Kamiuchi, S. and Hibino, Y. (2010). Protective effects of oral administrated ascorbic acid against oxidative stress and neuronal damage after cerebral ischemia/reperfusion in diabetic rats. *J. Health Sci.* **56**, 20-30.

Kozak, W. I. E. S., Conn, C. A. and Kluger, M. J. (1994). Lipopolysaccharide induces fever and depresses locomotor activity in unrestrained mice. *Am. J. Physiol.* **266**, R125-R135.

Kuzmin, I. V., Bozick, B., Guagliardo, S. A., Kunkel, R., Shak, J. R., Tong, S. and Rupprecht, C. E. (2011). Bats, emerging infectious diseases, and the rabies paradigm revisited. *Emerg. Health Threats J.* **4**, 7159.

Landmann, R., Scherer, F., Schumann, R., Link, S., Sansano, S. and Zimmerli, W. (1995). LPS directly induces oxygen radical production in human monocytes via LPS binding protein and CD14. *J. Leukoc. Biol.* **57**, 440-449.

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J. H., Wang, H., Crameri, G., Hu, Z., Zhang, H. et al. (2005). Bats are natural reservoirs of SARS-like coronaviruses. *Science* **310**, 676-679.

Lochmiller, R. L. and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**, 87-98.

Lorch, J. M., Meteyer, C. U., Behr, M. J., Boyles, J. G., Cryan, P. M., Hicks, A. C., Ballmann, A. E., Coleman, J. T. H., Redell, D. N., Reeder, D. M. et al. (2011). Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. *Nature* **480**, 376-378.

Maruoka, H. and Fujii, K. (2012). Effects of exercise and food consumption on the plasma oxidative stress. *J. Phys. Ther. Sci.* **24**, 37-41.

Meitern, R., Sild, E., Kilk, K., Porosk, R. and Hõrak, P. (2013). On the methodological limitations of detecting oxidative stress: effects of paraquat on measures of oxidative status in greenfinches. *J. Exp. Biol.* **216**, 2713-2721.

Moore, M. S. (2011). Ecological immunology in little brown myotis (*Myotis lucifugus*; Chiroptera) affected by white-nose syndrome. PhD dissertation. Boston University, Boston, MA, USA.

Müller, K., Voigt, C. C., Raila, J., Hurtienne, A., Vater, M., Brunnberg, L. and Schweigert, F. J. (2007). Concentration of carotenoids, retinol and α-tocopherol in plasma of six microchiroptera species. *Comp. Biochem. Physiol.* **147B**, 492-497.

Munshi-South, J. and Wilkinson, G. S. (2010). Bats and birds: exceptional longevity despite high metabolic rates. *Ageing Res. Rev.* **9**, 12-19.

Nussey, D. H., Pemberton, J. M., Pilkington, J. G. and Blount, J. D. (2009). Life history correlates of oxidative damage in a free-living mammal population. *Funct. Ecol.* **23**, 809-817.

Pearl, R. (1928). *The Rate of Living.* London: University of London Press.

Percival, S. S. and Sims, C. A. (2000). Wine modifies the effects of alcohol on immune cells of mice. *J. Nutr.* **130**, 1091-1094.

Perez-Rodriguez, L., Mougeot, F., Alonso-Alvarez, C., Blas, J., Viñuela, J. and Bortolotti, G. R. (2008). Cell-mediated immune activation rapidly decreases plasma

carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*). *J. Exp. Biol.* **211**, 2155-2161.
R Development Core Team (2010). *R: A Language and Environment for statIstical Computing*. Vienna, Austria: R Foundation for Statistical Computing. http://www.r-project.org
Reth, M. (2002). Hydrogen peroxide as second messenger in lymphocyte activation. *Nat. Immunol.* **3**, 1129-1134.
Rose, G., Passarino, G., Franceschi, C. and De Benedictis, G. (2002). The variability of the mitochondrial genome in human aging: a key for life and death? *Int. J. Biochem. Cell Biol.* **34**, 1449-1460.
Sacher, George A. (1959). Relation of lifespan to brain weight and body weight in mammals. *CIBA Foundation Colloquia on Aging* **5**, 115-141.
Schneeberger, K., Czirják, G. Á. and Voigt, C. C. (2013). Measures of the constitutive immune system are linked to diet and roosting habits of neotropical bats. *PLoS ONE* **8**, e54023.
Sghaier, H., Ghedira, K., Benkahla, A. and Barkallah, I. (2008). Basal DNA repair machinery is subject to positive selection in ionizing-radiation-resistant bacteria. *BMC Genomics* **9**, 297.
Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321.
Skibska, B., Józefowicz-Okonkwo, G. and Goraca, A. (2006). Protective effects of early administration of alpha-lipoic acid against lipopolysaccharide-induced plasma lipid peroxidation. *Pharmacol. Rep.* **58**, 399-404.
Sohal, R. S. and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. *Science* **273**, 59-63.
Sohal, R. S., Svensson, I. and Brunk, U. T. (1990). Hydrogen peroxide production by liver mitochondria in different species. *Mech. Ageing Dev.* **53**, 209-215.
Speakman, J. R. and Selman, C. (2011). The free-radical damage theory: accumulating evidence against a simple link of oxidative stress to ageing and lifespan. *Bioessays* **33**, 255-259.
Tolmasoff, J. M., Ono, T. and Cutler, R. G. (1980). Superoxide dismutase: correlation with life-span and specific metabolic rate in primate species. *Proc. Natl. Acad. Sci. USA* **77**, 2777-2781.
Victor, V. M., Rocha, M. and De la Fuente, M. (2004). Immune cells: free radicals and antioxidants in sepsis. *Int. Immunopharmacol.* **4**, 327-347.
Wibbelt, G., Moore, M. S., Schountz, T. and Voigt, C. C. (2010). Emerging diseases in Chiroptera: why bats? *Biol. Lett.* **6**, 438-440.
Wilhelm Filho, D., Althoff, S. L., Dafré, A. L. and Boveris, A. (2007). Antioxidant defenses, longevity and ecophysiology of South American bats. *Comp. Biochem. Physiol.* **146C**, 214-220.
Wilkinson, G. S. and South, J. M. (2002). Life history, ecology and longevity in bats. *Aging Cell* **1**, 124-131.
Wood, J. L., Leach, M., Waldman, L., Macgregor, H., Fooks, A. R., Jones, K. E., Restif, O., Dechmann, D., Hayman, D. T., Baker, K. S. et al. (2012). A framework for the study of zoonotic disease emergence and its drivers: spillover of bat pathogens as a case study. *Philos. Trans. R. Soc. B* **367**, 2881-2892.