Eco-Immunology and Oxidative Stress of Neotropical Bats

Dissertation zur Erlangung des akademischen Grades des
Doktors der Naturwissenschaften (Dr. rer. nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie
der Freien Universität Berlin

vorgelegt von

Karin Schneeberger
aus Sumiswald, Schweiz

2013

Die Dissertation wurde am Leibniz Institut für Zoo- und Wildtierforschung in Berlin im Zweitraum vom 15. September 2009 bis 20. August 2013 unter der Leitung von PD Dr. Christian C. Voigt angefertigt und am Institut für Biologie der Freien Universität Berlin eingereicht.

1. Gutachter: PD Dr. Christian C. Voigt
2. Gutachter: Prof. Dr. Silke Kipper

Disputation am: 9.12.2013

This thesis is based on the following manuscripts

1. Schneeberger, K.[1, 2], Czirják G.Á.[3] and Voigt, C.C.[1, 2] (2013) **Measures of the constitutive immune system are linked to diet and roosting habits of Neotropical bats.** PLoS ONE 8(1): e54023. (http://dx.doi.org/10.1371/journal.pone.0054023)

2. Schneeberger, K.[1, 2], Czirják G.Á.[3] and Voigt, C.C.[1, 2] (submitted) **Immunological parameters are linked to age and survival in a long-lived mammal, the greater sac-winged bat (*Saccopteryx bilineata*)**

3. Schneeberger, K. [1, 2], Czirják G.Á.[3] and Voigt, C.C.[1, 2] (2013) **Inflammatory challenge increases measures of oxidative stress in a free-ranging, long-lived mammal.** Journal of Experimental Biology 216, 4514-4519. (http://dx.doi.org/10.1242/jeb.090837)

4. Schneeberger, K. [1, 2], Czirják G.Á.[3] and Voigt, C.C.[1, 2] (submitted) **Frugivory reduces measures of oxidative stress in free-ranging bats**

[1] *Leibniz Institute for Zoo and Wildlife Research, Department Evolutionary Ecology, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany*

[2] *Freie Universität Berlin, Department Animal Behaviour, Takustr. 3, 14195 Berlin, Germany*

[3] *Leibniz Institute for Zoo and Wildlife Research, Department Wildlife Diseases, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany*

Content

Zusammenfassung

Langlebigkeit ist speziell für Tierarten mit langsamen Reproduktionsraten wichtig, da bei längerer Lebensdauer mehr Jungtiere produziert werden können, was die allgemeine Fitness steigert. Das Immunsystem ist dabei von zentraler Bedeutung, da es ein Individuum mit potenten Abwehrmechanismen gegen Pathogene ausstattet und so das Überleben bei Infektionen sichert. Verschiedene biotische und abiotische Faktoren spielen bei der Variabilität von Immunkomponenten eine wichtige Rolle, was durch Studien an Labor- und Zootieren bereits gezeigt werden konnte. Jedoch ist bisher nahezu unbekannt, inwiefern sich diese Erkenntnisse auf freilebende Säugetierpopulationen unter natürlicher Selektion ausweiten lassen.

Fledermäuse sind besonders geeignete Modelorganismen um öko-immunologische Fragestellungen anzugehen. Sie sind einerseits ökologisch höchst divers und andererseits dafür bekannt, Träger diverser Pathogene zu sein, gegen welche das Immunsystem entsprechend angepasst sein sollte. In **Kapitel I** beschäftigte ich mich mit der Frage, ob zwei ökologische Faktoren – Nahrungsnische und Hangplatz – mit Aspekten des Immunsystems von Fledermäusen verbunden sind. Mithilfe phylogenetisch korrigierter statistischer Methoden fand ich heraus, dass die Anzahl an Immunzellen zwischen Arten, die unterschiedliche Nahrungsnischen besetzen, variiert. Zudem gab es eine Verbindung zwischen der bakterizide Wirkung des Plasmas und Merkmalen der artspezifischen Hangplätze. Aufgrund meiner Ergebnisse schlussfolgere ich, dass diese ökologischen Faktoren womöglich eng mit dem Vorkommen bestimmter Pathogene verknüpft sind, und die evolutionäre Anpassung von zellulären und humoralen Immunkomponenten bei Säugetieren entsprechend durch deren jeweilige Ökologie beeinflusst wird.

Obwohl sich das Immunsystem einerseits positiv auf das Überleben auswirken kann, so ist es andererseits auch mit energetischen Kosten verbunden. Dies kann zu Konflikten mit anderen energieintensiven fitnessbestimmenden „Life-history“-Merkmalen führen und die Lebensspanne potenziell eher reduzieren statt verlängern. In **Kapitel II** untersuchte ich, wie verschiedene Immunkomponenten mit dem Überleben und Alter bei der großen Sackflügelfledermaus (*Saccopteryx bilineata*) zusammenhängen. Ich fand heraus, dass Tiere mit einer besonders hohen bakteriziden Wirkung des Plasmas und einer hohen Anzahl an Immunzellen aus den Populationen verschwanden. Entsprechend hatten ältere Tiere weniger Immunzellen als junge Tiere, woraus ich schließe, dass übermäßig erhöhte Immunfunktionen einer negativen Selektion unterliegen könnten.

Es stellte sich die Frage, welche Aspekte so negativ mit einer Immunantwort verbunden sein könnten, dass sie die Lebensspanne reduzieren. Eine Möglichkeit ist, dass eine Immunantwort vermehrt zu Schäden durch oxidativen Stress führen kann. Oxidativer Stress ist ein Ungleichgewicht zwischen reaktiven Sauerstoffspezies, die als schädliche Nebenprodukte während der aeroben Atmung produziert werden, und neutralisierenden Antioxidantien. Immunzellen produzieren Sauerstoffspezies zu Signalzwecken und um Pathogene direkt abzutöten. In **Kapitel III** testete ich daher, ob eine zelluläre Immunantwort den oxidativen Stress bei Fledermäusen verstärkt. Ich injizierte Lipopolysaccheride in 20 Brillenblattnasen (*Carollia perspicillata*) und konnte nach 24 Stunden eine zelluläre Immunantwort feststellen. Damit verbunden war eine erhöhte Konzentrationen reaktiver Sauerstoffmetabolite im Plasma, wobei die Konzentration von Antioxidantien gleich blieb. Bei Tieren, welche Kochsalzlösung injiziert bekamen, ergab sich keine Änderung in der Konzentration von Sauerstoffmetaboliten oder Antioxidantien. Zusätzlich stellte ich einen positiven generellen Zusammenhang zwischen der Anzahl an Immunzellen und reaktiven Sauerstoffmetaboliten fest. Daraus schließe ich, dass eine zelluläre Immunantwort den oxidativen Stress bei Fledermäusen erhöhen kann.

Oxidativer Stress kann ebenso wie Immunkomponenten mit ökologischen Faktoren zusammenhängen. Im Speziellen sollte die Konzentration von Antioxidantien bei einer antioxidantienreichen Nahrung ansteigen. Davon ausgehend verglich ich in **Kapitel IV** den oxidativen Stress von Fledermäusen mit unterschiedlicher Ernährungsweise und fand heraus, dass Früchtefresser eine geringere Konzentration reaktiver Sauerstoffspezies aufweisen als Arten, welche sich nicht von Früchten ernähren, obwohl die gemessen Antioxidantienkonzentration bei allen Arten ähnlich war. Womöglich haben Früchtefresser einen evolutionären Vorteil gegenüber Nicht-Früchtefressern, was teilweise die frühe Ausbreitung von Fledermausarten auf verschiedene Nahrungsnischen erklären könnte. Verglichen mit ähnlich großen, terrestrischen Säugetieren wiesen Fledermäuse in meiner Studie zudem einen geringeren oxidativen Stress auf. Da oxidativer Stress als Hauptgrund für Alterungsprozesse angesehen wird, könnte ein geringer oxidativer Stress die außergewöhnliche Langlebigkeit von Fledermäusen erklären.

Zusammenfassend betonen meine Studien, dass es wichtig ist, Methodiken, welche man bisher hauptsächlich bei Labor- und Zootieren angewandt hat, auf freilebende Populationen auszuweiten, um weitreichende Erkenntnisse über die evolutionäre Ökologie physiologischer Merkmale zu erhalten.

Summary

Survival and longevity are two important fitness parameters for species with low reproductive rates, as prolonged survival increases lifetime reproductive success and thus overall fitness. The immune system ensures an individuals ability to fight parasite and pathogens and is therefore of central importance for survival. It is hypothesised that variations in immune components can largely be attributed to the biotic and abiotic environment an animal lives in. Although comparative studies on captive populations found a connection between social and ecological factors with the immune system, evidence from natural populations are scarce.

Bats are ideal model organisms to investigate eco-immunological questions, as they are on one hand ecologically highly diverse, covering a wide variety of niches, and are on the other hand known to carry prominent pathogens, and thus their immune system should be adapted accordingly. In **Chapter I**, I asked if two ecological factors – diet and roost choice – are connected with aspects of the immune system in bats. Applying phylogenetic comparative methods, I found that white blood cell (WBC) counts of 24 Neotropical bat species varied with the species' diet, and that bacterial killing ability of the plasma (BKA) was connected to the permanence and protection of daytime roosts. Both ecological factors may mirror the pathogenic environment a species lives in, and thus these results suggest that ecology is an important factor in the evolution of immune components.

Although the immune system is of central importance for survival, mounting and maintaining immune components is energetically costly and thus can cause trade-offs with other life-history traits, leading to a reduction rather than prolongation of lifespan. In **Chapter II**, I investigated if survival and age is connected to immune components in the greater sac-winged bat (*Saccopteryx bilineata*). As this species shows a high roost fidelity, individuals disappearing from colonies after maturation most likely died. I assessed total WBC counts, BKA and immunoglobulin G titers in individuals of various age classes and observed which individuals remained in the colony after sampling. I found individuals with high BKAs and WBC counts to disappear from the colonies and in accordance with this finding, older individuals had lower WBC counts than young ones. This study gives first evidence that elevated levels of immune components may be associated with increased mortality risk in mammals and thus may to a certain extent be selected against.

With these results, the question arises what physiological processes connected to mounting an immune response can have negative effects on longevity. One potential

candidate is oxidative stress, an imbalance between reactive oxygen species (ROS) produced during normal energy production in aerobial organisms, and antioxidants that mitigate the negative effects of these ROS. During an immune response, the production of ROS should increase, as immune cells produce ROS for signalling and to directly kill pathogens. In **Chapter III**, I asked if mounting an immune response leads to an increase in measures of oxidative stress in bats. I injected 20 short-tailed fruit bats (*Carollia perspicillata*) with the antigen lipopolysaccharide, which caused a detectable cellular immune response after 24h. These individuals also showed an increase in reactive oxygen metabolites (ROM) measured in plasma, which are connected to the total ROS produced. The concentration of antioxidants did not change pre- and post-injection, which is why I concluded that even a potential short-term increase of antioxidants is not sufficient to mitigate the negative effects of elevated ROS levels. In control individuals injected with saline solution, I did not find any change in measures of oxidative stress and no cellular immune reaction was detected. I furthermore found that the general number of immune cells is positively correlated with levels of ROM. Therefore, mounting a cellular immune response may indeed increase oxidative stress in bats.

Likewise as found for immunological parameters, oxidative stress may be connected to ecological factors. For example, feeding on antioxidant rich diets such as fruits may increase the total antioxidant concentration and thus reduce oxidative stress. In **Chapter IV**, I therefore asked if measures of oxidative stress are connected to diet in bats. Although plasma antioxidant levels did not differ among feeding habits in 13 Neotropical bat species, the level of ROM was lower in frugivores than in other species. Potentially, frugivorous bats ingest more dietary antioxidants than non-frugivorous bats. I speculate that switching from insectivory to frugivory may have led to a fitness advantage in frugivorous bats, facilitating the radiation of bats to various ecological niches. Furthermore, I found that bats in general have relatively low levels of ROM and high antioxidant concentrations compared to similar-sized terrestrial mammals, which may account for the exceptional longevity of bats.

To conclude, these studies provide first insights into eco-immunology and oxidative stress in free-ranging mammals, highlighting the importance of transferring methods that were so far restricted to laboratory organisms or captive mammals to populations under natural selection in order to understand the evolutionary ecology of essential physiological traits.

General Introduction

Survival and longevity are amongst the most important fitness parameters for animals with a slow pace of life, as prolonged survival may increase lifetime breeding success, leading to a higher overall fitness. Thus, it is of central interest for evolutionary ecologists to understand the evolved adaptive mechanisms that shape the ability of an individual to survive to adulthood and thereafter. In my doctoral thesis, I focus on two aspects connected to survival and longevity – the immune system and oxidative stress – using Neotropical bats as free-ranging mammalian model organisms.

Eco-immunology

The immune system is one of the most important fitness-relevant traits ensuring survival, as it builds an individual's ability to fight against parasites and pathogens, which impose strong selective pressure on their hosts by reducing host fitness (Lehmann 1993). Hosts with a better immune defence suffer less from pathogens than individuals with low immunocompetence (e.g. Saino et al. 1997). Although mounting and maintaining immune functions may increase survival, immune components are energetically costly (Lochmiller and Deerenberg 2000) and thus immune functions can not be maximised at all times. Organisms therefore face a trade-off between immune functions and other life-history traits when resources are limited (Zuk and Stoehr 2002).

The immune system components can be grouped into four categories, depending on their form and function (Fig 1): Both cellular and humoral immunity are composed of innate components that are non-specific and form a first line of defence, and adaptive components that are specific to antigens and form an immunological memory after exposure to pathogens.

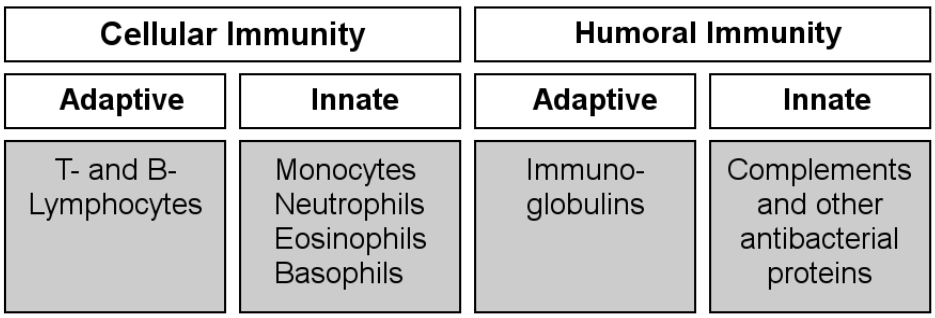


Fig. 1: Components of the immune system, sorted by form (cellular, humoral) and function (adaptive, innate).

Variations in components of the immune system have lately caught the attention of researchers from disciplines other than immunology. As a consequence, the research fields of physiology and ecology, two disciplines that hitherto worked independently, have recently formed the new field of ecological immunology, or eco-immunology for short (Schulenburg et al. 2009). Eco-immunology is the study of biotic and abiotic factors influencing the evolution of the immune system, aiming to understand what drives variation in immunity within and between species. By investigating aspects of eco-immunology, new insights can be gained in how physiology and ecology interact, and in further perspectives, how diseases evolve and host populations respond.

The studies by Nunn and his colleagues were the first to investigate how social and ecological factors are connected with immune components in mammals, comparing the constitutive cellular immune system among healthy captive primates and carnivores. Higher white blood cell counts were found in promiscuous species than in monogamous species (Nunn et al. 2000; Nunn 2002; Nunn et al. 2003), however other factors such as group size and diet failed to show a general connectivity to the cellular immune system in these two mammalian orders (Nunn 2002; Nunn et al. 2003).

Within-species variations in immune components may be caused chiefly by differences in life-history traits (Zuk and Stoehr 2002). It has been shown that the immune system may become impaired when resources are allocated mainly to reproduction (Christe et al. 2000; Christe et al. 2007). Investing energy in the immune system may not only increase the chance of survival, but could also in contrast decrease longevity by imposing costs to the host. For example, Eraud and colleagues (2009) triggered an immune response in Eurasian collared dove (*Streptopelia decaocto*) nestlings by injecting antigens from *Escherichia coli* and found that treated nestlings were more likely to be predated than control birds.

So far little attention has been paid to what factors influence immune components of free-ranging mammals. Possible trade-offs between immune function and survival of wild mammalian populations under natural selection are largely understudied. Studies on laboratory species and captive individuals may mask the effect of natural selection, which is why studies on free-ranging mammals are urgently needed to shed light on the functional relationship between the immune system and survival as well as interactions of ecological factors with immune components (Ricklefs and Wikelski 2002; Schulenburg et al. 2009).

Oxidative stress

Besides immune functions, other molecular processes can cause trade-offs with life-history traits such as longevity, for example oxidative stress. All aerobic organisms use oxygen to produce energy within the mitochondria of the cells. However, it is estimated that around 2% of oxygen consumed by cells is diverted to generate hydrogen peroxide and superoxide (Chance et al. 1979). These so-called reactive oxygen species (ROS) can lead to oxidative damage of cell components. ROS can cause peroxidation of membrane fatty acid chains, loss of sulfhydryls and carbonylation in proteins and modification of DNA (Sohal et al. 1990; Sohal & Weindruch 1996; Goyns 2002).

The negative effect of ROS can be mitigated by raising an antioxidant barrier, consisting of endogenous (e.g. enzymatic) and exogenous (diet-derived) antioxidants. Antioxidants convert ROS into less reactive molecules and thus prevent oxidative damaging of cell structures. An imbalance between ROS and antioxidants results in oxidative stress, which may impair the metabolism of an organism (Rose et al. 2002). Oxidative stress is therefore regarded as the main cause of ageing for cells and whole organisms (Harman 1955). In general, variations in longevity among species have been shown to correlate negatively with the amount of ROS 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). However, the free-radical theory of ageing has recently been under debate, as not all experimental and correlative studies support the hypothesis that high oxidative stress leads to lower life-spans (Speakman and Selman 2011; Selman et al. 2012).

The creation of ROS generally increases linearly with the amount of energy produced, i.e. with mass-specific metabolic rate (Pearl 1928; Harman 1955; Sacher 1959). Therefore, large mammals with relatively low basal metabolic rate usually live longer than small mammals with relatively high basal metabolic rate (Hulbert et al. 2007). The creation of ROS also increases during certain processes, such as the physiological response to pathogens. During an immune response, the host metabolic rate is usually elevated (Sheldon and Verhulst 1996), which leads to higher oxygen consumption rates connected with higher mitochondrial activity and consequently to an increased ROS production (Finkel and Holbrook 2000). Additionally, different white blood cell subtypes involved in immune responses produce ROS to directly kill pathogens (Droege 2002), and to enhance the activation of T-lymphocytes (Droege 2002; Reth 2002). Mounting an immune response

therefore is not only energetically costly, but may also lead to oxidative damage of cell components and thus early senescence of cells.

Bats immune system and oxidative stress

Bats are the second largest mammalian order, distributed over all continents except Antarctica, and the only mammals able of empowered flight. They occupy numerous ecological niches (Fig. 2) and provide important ecosystem services by dispersing seeds, pollinating plants and feeding on pest insects (Kunz et al. 2011).

During the past decades, bats have been drawing increasing attention because they host some of the most prominent zoonotic viral pathogens (Wibbelt et al. 2010; Wood et al. 2012), such as lyssaviruses (Kuzmin et al. 2011), paromyxoviruses (e.g. Hendra, Nipah; Drexler et al. 2012) and filoviruses (e.g. Ebola and Marburg; Monath 1999). Surprisingly, although carrying pathogens that are potentially lethal for humans and livestock, bats rarely show signs of suffering or sickness. However, the recently emerging white-nose syndrome (WNS), an infectious diseases caused by the fungus *Geomyces destructans*, has caused dramatic mass mortalities in North American bats (Blehert et al. 2009; Lorch et al. 2011), driving some populations to the edge of local extinction (Frick et al. 2010). Immune deficiency could facilitate the susceptibility for WNS (Moore 2011; Moore et al. 2011), which is why a better knowledge of the bat immune system could help to explain why bats vary largely in their susceptibility towards pathogens.

Surprisingly little is known about bat immunity. Within species, immune responses are known to vary between colonies and types of roosts (Allen et al. 2009), and a first comparative study on Neotropical bats has shown that the number of immune cells varies greatly among species, with one frugivorous species having the lowest white blood cell count ever encountered in mammals (Schinnerl et al. 2011). However, it is so far unclear what factors drive these differences, and whether aspects of the immune system are connected with survival and longevity in bats.

In general, bats are of particular interest for the study of survival and longevity, since the maximal recorded life span of bats is on average 3.5 times higher than those of similar sized terrestrial mammals (Austad and Fischer 1991; Wilkinson and South 2002; Brunet-Rossinni and Austad 2004). At the same time, the metabolic rates of bats are exceptionally high due to their ability of empowered flight (Munshi-South and Wilkinson 2010). Thus, in theory, bats should produce more ROS by consuming more oxygen than similar sized terrestrial mammals (Pearl 1928; Harman 1955; Sacher 1959). The exceptional longevity

of bats therefore seems to be somewhat paradox, and thus the question arises whether bats produce less ROS than similar sized terrestrial mammals or whether they have a particularly strong antioxidant barrier in order to mitigate negative effects of pro-oxidants.

Fig 2: Neotropical bat species encountered in the Caribbean lowland of Costa Rica, where the studies of my thesis have been conducted. Facial characteristics mirror the diversity of ecological niches bats cover in the Neotropics. From top left to bottom right: *Vampyressa pusilla, Lonchophylla robusta, Glossophaga commissarisi, Myotis elegans, Tonatia saurophila, Carollia castanea, Artibeus intermedius, Micronycteris microtis, Uroderma billobatum, Centronycteris centralis, Artibeus jamaicensis, Artibeus watsonii, Trachops cirrhosus, Rhogeessa io, Chiroderma villosum, Hylonycteris underwoodi, Carollia perspicillata, Sturnira lilium, Saccopteryx bilineata, Carollia sowelli, Mimon crenulatum, Molossus currentium, Desmodus rotundus, Phyllostomus hastatus, Phyllostomus discolor, Mesophylla macconnelli, Artibeus phaeotis, Ectophylla alba, Myotis albescens, Noctilio albiventris.*

Studies on oxidative stress in bats are scarce and confined to single species. Furthermore, they are rather inconsistent. For example, tissue of little brown bats (*Myotis lucifugus*) show higher concentrations of hydrogen peroxide, one of the most important ROS, than white-footed mice (*Peromyscus leucopus*), but lower concentrations than northern short-tailed shrews (*Blarina brevicauda*; Brunet-Rossinni 2004). At the same time, the activity of superoxide dismutase, an important enzymatic antioxidant, does not differ between *M. lucifugus, B. brevicauda* and *P. leucopus* (Brunet-Rossinni 2004). However, five South American bat species have been found to have higher levels of both enzymatic and non-enzymatic antioxidants in their organs than similar-sized terrestrial mammals (Wilhelm Filho et al. 2007). Thus, it remains unclear if bats in general differ in their oxidative stress from similar-sized terrestrial mammals and if this accounts for their high longevity. Furthermore, as for the immune system, it is still unknown what factors drive differences in oxidative stress in bats and mammals in general.

References

Adelman, Robert, Saul, Robert L., and Ames, Bruce N. (1988) Oxidative damage to DNA: relation to species metabolic rate and life span. *Proceedings of the National Academy of Sciences* **85**, 2706-2708.

Allen, L. C., Turmelle, A. S., Mendoca, 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*). *Journal of Comparative Physiology B* **179**, 315-323.

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

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

Blehert, David S., Hicks, Alan C., Behr, Melissa, Meteyer, Carol U., Berlowski-Zier, Brenda M., Buckles, Elizabeth L., Coleman, Jeremy TH, Darling, Scott R., Gargas, Andrea, and Niver, Robyn (2009) Bat white-nose syndrome: an emerging fungal pathogen? *Science* **323**, 227.

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

Brunet-Rossinni, A. K. and Austad, S. N. (2004) Ageing studies on bats: a review. *Biogerontology* **5**, 211-222.

Chance, Britton, Sies, Helmut, and Boveris, Alberto (1979) Hydroperoxide metabolism in mammalian organs. *Physiological Reviews* **59**, 527-605.

Christe, P., Arlettaz, R., and Vogel, P. (2000) Variation in intensity of a parasitic mite (*Spinturnix myoti*) in relation to the reproductive cycle and immunocompetence of its bat host (*Myotis myotis*). *Ecology Letters* **3**, 207-212.

Christe, P., Glaizot, O., Evanno, G., Bruyndonckx, N., Devevey, G., Yannic, G., Patthey, P., Maeder, A., Vogel, P., and Arlettaz, R. E. L. (2007) Host sex and ectoparasites choice: preference for, and higher survival on female hosts. *Journal of Animal Ecology* **76**, 703-710.

Drexler, J. F., Corman, V. M., Muller, M. A., Maganga, G. D., Vallo, P., Binger, T., Gloza-Rausch, F., Rasche, A., Yordanov, S., Seebens, A., Oppong, S., Sarkodie, Y. A., Pongombo, C., Lukashev, A. N., Schmidt-Chanasit, J., Stocker, A., Carneiro, A. J. B.,

Erbar, S., Maisner, A., Fronhoffs, F., Buettner, R., Kalko, E. K. V., Kruppa, T., Franke, C. R., Kallies, R., Yandoko, E. R. N., Herrler, G., Reusken, C., Hassanin, A., Kruger, D. H., Matthee, S., Ulrich, R. G., Leroy, E. M., and Drosten, C. (2012) Bats host major mammalian paramyxoviruses. *Nature Communications* **3**.

Droege, W. (2002) Free radicals in the physiological control of cell function. *Physiological Reviews* **82**, 47-95.

Eraud, Cyril, Jacquet, Anne, and Faivre, Bruno (2009) Survival cost of an early immune soliciting in nature. *Evolution* **63**, 1036-1043.

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

Frick, Winifred F., Pollock, Jacob F., Hicks, Alan C., Langwig, Kate E., Reynolds, D. Scott, Turner, Gregory G., Butchkoski, Calvin M., and Kunz, Thomas H. (2010) An emerging disease causes regional population collapse of a common North American bat species. *Science* **329**, 679-682.

Harman, D. (1955) *Aging: a theory based on free radical and radiation chemistry*. University of California Radiation Laboratory

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

Kunz, Thomas H., Braun de Torrez, Elizabeth, Bauer, Dana, Lobova, Tatyana, and Fleming, Theodore H. (2011). Ecosystem services provided by bats. *Annals of the New York Academy of Sciences* **1223**, 1-38.

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. *Emerging Health Threats Journal* **4**, 7159.

Lehmann, T. (1993). Ectoparasites: direct impact on host fitness. *Parasitology Today* **9**, 8-13.

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., and Reeder, D. A. M. (2011). Experimental infection of bats with Geomyces destructans causes white-nose syndrome. *Nature* **480**, 376-378.

Monath, T. P. (1999). Ecology of Marburg and Ebola viruses: Speculations and directions for future research. *Journal of Infectious Diseases* **179**, S127-S138.

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

Moore, M. S., Reichard, J. D., Murtha, T. D., Zahedi, B., Fallier, R. M., and Kunz, T. H. (2011). Specific alterations in complement protein activity of little brown myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. *Plos One* **6**, e27430.

Munshi-South, Jason and Wilkinson, Gerald S. (2010). Bats and birds: Exceptional longevity despite high metabolic rates. *Ageing Research Reviews* **9**, 12-19.

Nunn, C. L. (2002). A comparative study of leukocyte counts and disease risk in primates. *Evolution* **56**, 177-190.

Nunn, C. L., Gittleman, J. L., and Antonovics, J. (2000). Promiscuity and the primate immune system. *Science* **290**, 1168-1170.

Nunn, C. L., Gittleman, J. L., and Antonovics, J. (2003). A comparative study of white blood cell counts and disease risk in carnivores. *Proceedings of the Royal Society B* **270**, 347-356.

Pearl, R. (1928). *The rate of living*. University of London Press

Reth, M. (2002). Hydrogen peroxide as second messenger in lymphocyte activation. *Nature Immunology* **3**, 1129-1134.

Ricklefs, Robert E. and Wikelski, Martin (2002). The physiology/life-history nexus. *Trends in Ecology & Evolution* **17**, 462-468.

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? *International Journal of Biochemistry & Cell Biology* **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.

Saino, N., Bolzern, A. M., and Møller, A. P. (1997). Immunocompetence, ornamentation, and viability of male barn swallows (*Hirundo rustica*). *Proceedings of the National Academy of Sciences* **94**, 549.

Schinnerl, M., Aydinonat, D., Schwarzenberger, F., and Voigt, C. C. (2011). Hematological survey of common Neotropical bat species from Costa Rica. *Journal of Zoo and Wildlife Medicine* **42**, 382-391.

Schulenburg, H., Kurtz, J., Moret, Y., and Siva-Jothy, M. T. (2009). Introduction. Ecological immunology. *Philosophical Transactions of the Royal Society B* **364**, 3-14.

Selman, C., Blount, J. D., Nussey, D. H. and Speakman, J. R. (2012) Oxidative damage, ageing, and life-history evolution: where now? *Trends in Ecology & Evolution* **27**, 570-577.

Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution* **11**, 317-321.

Sohal, Rajindar S. and Weindruch, Richard (1996). Oxidative stress, caloric restriction, and aging. *Science* **273**, 59-63.

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, Julie M., Ono, Tetsuya, and Cutler, Richard G. (1980). Superoxide dismutase: correlation with life-span and specific metabolic rate in primate species. *Proceedings of the National Academy of Sciences* **77**, 2777-2781.

Wibbelt, Gudrun, Moore, Marianne S., Schountz, Tony, and Voigt, Christian C. (2010). Emerging diseases in Chiroptera: why bats? *Biology Letters* **6**, 438-440.

Wilhelm Filho, Danilo, Althoff, Sergio Luiz, Dafre, Alcir Luiz, and Boveris, Alberto (2007). Antioxidant defenses, longevity and ecophysiology of South American bats. *Comparative Biochemistry and Physiology C* **146**, 214-220.

Wilkinson, G. S. and South, J. M. (2002). Life history, ecology and longevity in bats. *Aging Cell* **1**, 124-131.

Wood, James L., Leach, Melissa, Waldman, Linda, MacGregor, Hayley, Fooks, Anthony R., Jones, Kate E., Restif, Olivier, Dechmann, Dina, Hayman, David T., Baker, Kate S., Peel, Alison J., Kamins, Alexandra O., Fahr, Jakob, Ntiamoa-Baidu, Yaa, Suu-Ire, Richard, Breiman, Robert F., Epstein, Jonathan H., Field, Hume E., and Cunningham, Andrew A. (2012). A framework for the study of zoonotic disease emergence and its drivers: spillover of bat pathogens as a case study. *Philosophical Transactions of the Royal Society B* **367**, 2881-2892.

Zuk, Marlene and Stoehr, Andrew M. (2002). Immune defense and host life history. *The American Naturalist* **160**, S9-S2.

Thesis Outline

Due to their high diversity, their prominence to carry infections diseases and their exceptional lifespan, bats are an ideal study system to investigate questions regarding the link between immunity, oxidative stress and longevity and the connectivity of these life-history aspects with ecological factors. The aim of this thesis was to investigate several aspects of eco-immunology and oxidative stress in Neotropical bats in order to shed new light on the evolutionary ecology of the immune system and ageing in mammals in general.

The results are presented in four manuscripts, comprised in the following chapters:

Chapter I: Measures of the constitutive immune system are linked to diet and roosting habits of Neotropical bats

Ecological factors are of central importance in the emergence and transmission of diseases and thus may shape the immune system of a species. In this study, I investigated several aspects of the constitutive cellular and humoral immune system of free-ranging Neotropical bats, asking whether these aspects are connected with diet and shelter, two ecological factors likely to influence transmission of parasites and pathogens. I found that white blood cell count differs between dietary niche and that roost choice is connected with the ability of the plasma to kill bacteria. Therefore, ecology may be an important factor in the evolution of the immune system in bats and mammals in general.

Chapter II: Immunological parameters are linked to age and survival in a long-lived mammal, the greater sac-winged bat (*Saccopteryx bilineata*)

Mounting and maintaining immune functions is energetically costly, causing potential trade-offs with other life-history traits such as longevity. In Chapter II, I investigated the connectivity of aspects of the immune system with age and survival in the greater sac-winged bat (*Saccopteryx bilineata*). I found that bats with more immune cells and higher ability of the plasma to kill bacteria are more likely to disappear from the populations than individuals with low white blood cell count and low bacterial killing ability. Consequently, old bats had fewer white blood cells than young ones. However, bacterial killing ability did not decrease with age. In Chapter II, I therefore provide first evidence for the connectivity of several aspects of the immune system with survival and age in a free-ranging mammal.

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

Oxidative stress should increase during an immune response, leading to higher damage on cell components. Therefore, oxidative stress may be of central importance for long-lived species that are known to carry various parasites and pathogens, such as bats. In Chapter III, I explored if an immune challenge increases oxidative stress in the short-tailed fruit bat (*Carollia perspicillata*). I found that, although antioxidant concentration remained the same, the concentration of reactive oxygen metabolites that are directly connected to the total reactive oxygen species produced increased during a cellular immune response in experimental individuals injected with bacterial derived lipopolysaccharides. There was no increase in reactive oxygen metabolites or antioxidants in control animals injected with saline solution that did not show an immune response. Chapter III therefore shows that fighting an infection may lead to an increase in oxidative stress in bats.

Chapter IV: Frugivory reduces measures of oxidative stress in free-ranging bats

Oxidative stress may be connected to the ecological niche of an animal, as for example feeding on diets with high concentration of antioxidants should reduce oxidative stress. In this study, I investigated if measures of oxidative stress varies among dietary niches of 13 Neotropical bat species. I found that although antioxidant levels did not differ between species, the concentration of reactive oxygen metabolites in plasma was reduced in bats that fed on fruits compared to species that only partly or to no extent incorporated fruits in their diet. Furthermore, I found that bats in general have a lower concentrations of reactive oxygen metabolites and higher levels of plasma antioxidants than similar sized, terrestrial mammals. Chapter IV therefore comprises first evidence that measures of oxidative stress varies among dietary niches in mammals.

Chapter I

Measures of the constitutive immune system are linked to diet and roosting habits of Neotropical bats

(Published in PLoS ONE)

OPEN ACCESS Freely available online

PLOS ONE

Measures of the Constitutive Immune System Are Linked to Diet and Roosting Habits of Neotropical Bats

Karin Schneeberger[1,2]*, Gábor Á. Czirják[1], Christian C. Voigt[1,2]

1 Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany, **2** Freie Universität Berlin, Department Animal Behaviour, Berlin, Germany

Abstract

Ecological and social factors are central in the emergence and transmission of infectious diseases, thus bearing the potential for shaping a species' immune functions. Although previous studies demonstrated a link between social factors and the cellular immune system for captive mammals, it is yet poorly understood how ecological factors are connected with the different branches of the immune system in wild populations. Here, we tested how variation in aspects of the constitutive cellular and humoral immune system of free ranging bats is associated with two ecological factors that likely influence the putative risk of species to become infected by parasites and pathogens: diet and shelter. We found that white blood cell counts of 24 syntopic Neotropical bat species varied with the species' diet and body mass. Bats that included at least partially vertebrates in their diet exhibited the highest white blood cell counts, followed by phytophagous and insectivorous species, which is in agreement with the assumption that the immune system varies with the pathogen transmission risk of a trophic level. The soluble part of the constitutive immune response, assessed by an *in vitro* bacterial killing assay, decreased with increasing roost permanence. Our results suggest that the ecology is an important factor in the evolution of the immune system in bats and probably also other mammals.

Citation: Schneeberger K, Czirják GÁ, Voigt CC (2013) Measures of the Constitutive Immune System Are Linked to Diet and Roosting Habits of Neotropical Bats. PLoS ONE 8(1): e54023. doi:10.1371/journal.pone.0054023

Editor: Justin David Brown, University of Georgia, United States of America

Received August 8, 2012; **Accepted** December 7, 2012; **Published** January 14, 2013

Funding: This study was supported by funds from the Leibniz Institute for Zoo and Wildlife Research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: schneeberger@izw-berlin.de

Introduction

The immune system provides animals with a set of cellular and molecular defence mechanisms against potentially infectious agents. Parasites and pathogens impose strong selective pressure on their hosts by reducing, for example, host fitness [1]. Social and ecological factors that are linked to parasite and pathogen transmission risks are therefore most likely influencing the immune system of mammals. Previous studies have shown that high population densities and large group sizes of hosts may increase the risk for horizontal transmission of pathogens due to frequent encounters between infected and healthy individuals [2–4]. For example, pathogen prevalence increases with increasing group size in both birds and mammals [5–8]. Similarly, ecological factors such as feeding habits have been shown to influence the pathogen transmission risk when vertebrates are likely to ingest contaminated food or infected prey [9,10]. Therefore, both social and ecological factors may affect pathogen transmission risk among animals, and as a consequence the immune competence of animals should be related to these factors. Hosts with a better immune defence are expected to suffer less from pathogens, as has been shown in barn swallows (*Hirundo rustica*), where individuals with a stronger immune response survive better than less immune competent individuals [11]. However, as mounting, using and maintaining an effective immune response is energetically costly [12], individuals may have to trade these costs against other life-history components such as growth and reproductive output [13]. Therefore, the relative threat of infection resulting from the putative pathogen transmission risk of an ecological niche should be mirrored by the immune competence: species with a low infection risk should maintain a relatively low basal immune competence compared to species with high infection risk in order to minimise physiological and behavioural trade-offs.

Nunn and colleagues were the first to investigate social and ecological factors influencing the immune system of mammals by comparing the cellular constitutive immune system among healthy captive primates and carnivores. They showed that promiscuous species have higher white blood cell counts than monogamous species, probably due to an increased risk of acquiring sexually transmitted diseases [14–16]. However, other factors such as group size and diet failed to show general correlations with the cellular immune system [15,16]. Only eosinophils increased with the percentage of meat in the diet of carnivores [16]. Additionally, while Nunn and colleagues used data obtained from healthy captive wildlife species, inter-specific studies on free-ranging mammals regarding the influence of ecological factors on the immune system are still lacking. In contrast to free-ranging animals, captive animal populations may face a lowered disease risk when managed by veterinarians and nutritional experts. Furthermore, while the cellular aspect of the constitutive immune system has already been investigated [15,16], studies on the soluble immune factors are restricted to intra-specific studies only (e.g. [17,18]).

Here, we investigated aspects of both the cellular and soluble part of the constitutive immune system of free-ranging mammals and ask whether their variation is associated with ecological factors. Bats are an ideal study group to investigate these questions,

as they not only occupy numerous ecological niches, but are also associated with a number of emerging infectious diseases [19,20], including the fungus *Geomyces destructans*, the causative agent of the White-Nose Syndrome, which recently lead to dramatic declines in North American bat populations [21]. Specific mechanisms of disease transmission are poorly understood in bats, yet some studies suggest horizontal transmission of pathogens via contaminated food. For example, Nipah viruses are probably transmitted via fruits contaminated by faeces, urine or saliva of frugivorous bats [22,23], and rabies viruses are known to be transmitted when sanguinivorous vampire bats feed on their prey [24]. Bats cover a wide range of trophic levels, ranging from nectarivory, frugivory, omnivory, insectivory to carnivory. Trophic levels may vary with respect to their pathogen transmission risk according to the likelihood of animals to consume food items contaminated with infective agents and parasites. We therefore expected that feeding habits of bats influence their immune system according to the relative threat of infection with bacteria, viruses and parasites by oral-faecal route or by direct contact. Sanguinivorous species and species feeding at least partially on vertebrates (hereafter called carnivorous bats) face pathogen transmission risk due to direct contact with closely related prey species, while phytophagous species (frugivores and nectarivores) may feed on food sources that are potentially contaminated by faeces and saliva. Insects are important intermediate hosts for pathogens such as haemoparasites, different bacterial and viral agents, which may as well impose a selective pressure on the immune system of insectivorous bats. We therefore hypothesise that investment in the cellular immune system and the bacterial killing ability, as a measure of the constitutive innate immune system, will vary between species of different trophic levels.

Besides their diversity in dietary niches, bats also use a variety of structures as daytime shelters [25]. Patterson and colleagues [26] found that bat species roosting in more permanent and protected sites have a higher ectoparasite load than species using more ephemeral structures, highlighting that shelters vary in pathogen transmission risk. Furthermore, bacteria, viruses and parasites may be more abundant in roosts that are well protected from environmental factors such as precipitation. Thus, bats may face an increased risk of infection when using sheltered structures. Accordingly, we hypothesise that immune function should co-vary with shelter type. Therefore, we predict that species roosting in more permanent sites, presumably with higher risk of infection, would exhibit greater investment in the cellular and soluble parts of the constitutive immune system as compared with species roosting in more ephemeral structures.

In this study, we investigated aspects of both the cellular and soluble part of the constitutive immune system of 24 free-ranging Neotropical bat species. We used blood smears to estimate number of total and differential WBC counts, a method which has been used in birds and bats before [27,28]. WBC count is characteristic for cell-mediated processes in response to infections and can been used as an indirect measure of an individual's investment in cellular immune defence. Additionally, we extracted plasma from each individual for measuring the *in vitro* bacterial killing activity (BKA) mediated by the complement and other antibacterial proteins. Thus, we obtained quantitative measurements of two major parts of the constitutive immune system of each individual, which can then be averaged on the species level and compared to the mean of other species with respect to diet and shelter permanence and protection.

Materials and Methods

Ethic Statement

This study was approved by the institutional animal welfare and ethics committee of the Leibniz Institute for Zoo and Wildlife Research (permit number: 2011-08-01). Sample collection was authorised by the Ministerio del Ambiente y Energia (MINAE; permit number 163-20911-SINAC) of Costa Rica and complied with the current laws of the country.

Blood Sampling

We collected samples from 178 individuals belonging to 24 bat species at "La Selva" Biological Station (10°25′N; 84°00′W, Province Heredia, Costa Rica) in November and December 2010 by catching bats between 5 pm and 10 pm at ground level using nylon mist nets (2.5 m height, Ecotone, Gdynia, Poland). Species were identified according to Timm and LaVal [29]. As it is not possible to distinguish between *Artibeus phaeotis* and *Artibeus watsoni* in the field, we will refer to these two species as *Artibeus watsoni c.f.* [30]. *Saccopteryx bilineata* were caught at dawn (5 am–7 am) when individuals returned to their daytime roost. Captured individuals were weighed with a spring balance (accuracy 0.5 g, Pesola balance; Switzerland). Sex, age and reproductive status were assessed. Juveniles were distinguished from adults by examining the degree of the epiphysial closure of the phalanges. Pregnant and lactating bats as well as juveniles were releases immediately at the site of capture. From all other bats, a small blood sample of no more than 5% of the total blood volume was taken by punctuating the antebrachial vein with a sterile needle and collecting the blood droplets with a heparinised capillary. A subsample of the blood was taken to prepare a blood smear on glass slides (Microscope Slides (76×26 mm), cut edges, Menzel, 38116 Braunschweig, Germany). The plasma was collected after centrifugation and stored at −80°C until further analysis. All bats were released at the site of capture.

White Blood Cell Counts

Blood smears were stained with May-Gruenwald's solution (#T863.2, Carl Roth GmbH) and Giemsa (#T862.1, Carl Roth GmbH). Total WBC count for each individual was estimated manually by taking the mean of 10 visual fields, counting the cells with a microscope under 200× magnifications. Some former publications using the same method multiplied this mean with a certain species-specific constant to obtain the number of white blood cells per microliter (e.g. [31,32]). As no such constant is known for bats yet, and multiplication would not change the relative differences among species, we used the mean number of leukocytes per visual field for statistical analysis.

Additionally, after estimating the total WBC counts from blood smears, we validated this method by checking for correlation between our data obtained from blood smears and data obtained with a conventional method (Unopette^{TM} capillary system) of the same species at the same site in a previous year [30], finding a strong positive correlation (linear model; $R^2 = 0.803$; $N = 12$; $t = 7.06$; $p < 0.001$).

Differential white blood cell (DWBC) counts were performed counting 100 leukocytes under 1000× magnification (oil immersion) and calculating the relative numbers of lymphocytes, monocytes, neutrophils, basophils and eosinophils. Absolute numbers of the different leukocytes were calculated by multiplication with total WBC counts.

Bacterial Killing Activity

We measured the soluble aspect of the constitutive innate immunity by assessing the *in vitro* bacterial killing activity of the plasma against *Escherichia coli* [33] following the method of Tieleman and colleagues [34]. This assay has been used previously on different free-ranging and captive wild species, including bats [17,33,35]. Plasma samples were diluted 1:20 in CO_2-independent media (#18045, Gibco-Invitrogen, CA), enriched with 4 mM L-Glutamine (#25030, Gibco-Invitrogen, CA) and 5% Fetal Calf Serum (#S0115, Biochrom AG). To each diluted sample (140 µl) we added 10 µl of a suspension of live *E. coli* (ATCC #8739). The bacterial suspension was adjusted to a concentration of ~200 colonies per 50 µl of diluted plasma-bacteria mixture. After incubation, for 30 min at 37°C (mammalian body temperature), 50 µl of the plasma-bacteria mixture was spread aliquots onto Tryptic Soy Agar plates (#CP70.1, Carl Roth GmbH) in duplicate, and the plates were incubated overnight at 37°C. To obtain the initial number of bacteria that we had before starting to interact with the plasma, we diluted 140 µl media alone with bacterial suspension and plated immediately. On the following day the colony-forming units were counted and the bacterial killing activity was defined as the percent of the killed bacteria, which was calculated as 1– (average of the viable bacteria after incubation/ the initial number of bacteria). The average was calculated from two plates per sample.

Storage time of the samples can have an influence on BKA [36]. The storage time of our samples ranged from 41 to 81 days, with a mean of 63 days. We therefore tested the effect of storage time on BKA, and could not find an effect (Spearman rank correlation; $rho = -0.062$; $p = 0.442$; $n = 158$). Also, we use species mean for our analysis and as individuals of one species were not all captured nor analysed on the same day, the mean storage time of the samples of one species should be approximately the same. We tested this assumption and found no evidence against it, as there was no correlation between mean storage time and mean BKA of the species (Spearman rank correlation; $rho = -0.120$; $p = 0.576$; $n = 24$).

Data Sources from Literature and Statistical Analysis

All bat species were assigned to a dietary niche according to LaVal and Rodríguez [37]: insectivory, phytophagy and carnivory. Phytophagous species included nectarivorous and frugivorous bats, while vampire bats (*Desmodus rotundus*) and species feeding occasionally on vertebrates (*Trachops cirrhosus* and *Phyllostomus hastatus*) were summarised as carnivorous bats. Roosts were categorised after Patterson and colleagues [26]. They established six categories of roost ranks based on logarithmic differences in their estimated durability, with 1 representing the most ephemeral and least protected roost (e.g. rolled leaves and foliage) and 6 the most permanent and protected roost (e.g. caves). As some bat species are known to use different roost types, intermediate ranks were calculated for these species, weighing each roost according to the order in which they were listed in the literature used by Patterson and colleagues (e.g. with three roosts ranked 6, 6, and 5, the weighted rank was calculated as $(3\times6+2\times6+1\times5)/6 = 5.83$), leading to a continuous scaling of the variable "roost" [26]. Table 1 reports the roost category assigned to each bat species included in this study.

Data on other social and ecological factors such as age, group size and mating system were only available for as small subsample of the species. Therefore, we could not include these factors in our analysis.

We used "R" version 2.13.1 for all statistical analysis [38]. Mean body mass, WBC (total and differential) counts and BKA was calculated for all species. As two closely related species may share inherited characteristics from a common ancestor, species-specific data can not be considered as statistically independent [39]. We therefore calculated phylogenetic generalised least squares models (PGLS [40]) on the effect of dietary niche, roost use and body mass on total and differential WBC count as well as on BKA using the "gls" function of the package "nlme" [41] and accounted for phylogeny using the "correlation" function of the package "ape" [42]. We used a phylogenetic tree modified after Jones and colleagues [43] (Fig. 1). Details of the phylogeny of the genus *Carollia* was drawn from Hoffmann & Baker [44]. We used the covariance matrix "corGrafen" [45], as in initial trials, this resulted in the lowest estimates of model AIC (Akaike's information criterion). Accordingly, as branch lengths were unknown, we artificially computed them as suggested by Grafen, with the length of a branch being the number of descending taxa minus 1 [45]. As the number of individuals caught for each species varies greatly, we weighted the data by sample size to account for heteroscedasticity of variance. Residuals of the models were normally distributed, except for lymphocytes, where we applied log-transformation. For all analyses, we set the level of significance to $\alpha = 0.05$.

Results

We assessed both WBC counts and BKAs in samples from 154 out of 178 captured bats. From the other 24 individuals, we obtained sample volumes that were only sufficient for either WBC or BKA. In 20 out of these 24 individuals, we counted WBCs, and in 4 we measured the BKA. Table 1 reports the mean and standard error of the mean (SEM) for body mass, total WBC counts and BKA as well as the sample sizes for each species. Details on differential WBC counts are given in Table S1 of the electronic supplement.

Total WBC counts increased significantly with body mass ($t = 5.4$; $F_{1,19} = 29.2$; $p<0.001$; Fig. 2A), and species differed significantly in their total number of WBCs according to their diet ($F_{2,19} = 7.0$; $p = 0.005$; Fig. 2B): Carnivorous bats had significantly higher WBC counts than insectivorous species ($t = 3.2$; $p = 0.005$), and phytophagous species had significantly more WBCs than insectivorous species ($t = 2.3$; $p = 0.036$). There was no difference between phytophagous and carnivorous bats ($t = 1.1$; $p = 0.27$). We found no association between WBC count and roost category ($t = 1.6$; $F_{1,19} = 2.7$; $p = 0.119$; Fig. 2C).

The association of the number of different WBCs with ecological factors depended on the type of WBCs (Table 2). While lymphocytes and basophils were associated with roost permanence and protection, monocytes and eosinophils differed in number between bats with different dietary niches. Carnivorous bats had higher numbers of cells than insectivorous bats (monocytes: $t = 2.3$; $p = 0.030$; eosinophils: $t = 3.3$; $p = 0.004$). The number of eosinophils were higher in carnivorous than phytophagous bats ($t = 2.6$; $p = 0.017$), while there was no significant difference in monocytes ($t = 0.9$; $p = 0.388$). Insectivorous and phytophagous species did not differ in the number of cells (monocytes: $t = 1.6$; $p = 0.119$; eosinophils: $t = 0.21$; $p = 0.836$).

Roost choice of bat species influenced plasma BKA: Species roosting in more ephemeral roosts had lower BKAs that species roosting in permanent roosts ($t = -2.8$; $F_{1,19} = 7.6$; $p = 0.013$; Fig. 2F). There was no significant effect of body mass ($t = 0.09$; $F_{1,19}<0.01$; $p = 0.927$; Fig. 2D) or diet ($F_{2,19} = 0.29$; $p = 0.754$; Fig. 2E) on BKA.

Table 1. Mean and standard error of the mean (SEM) for body mass, white blood cell count (WBC) and bacterial killing activity (BKA) of 24 Neotropical bat species.

Species	Body mass (g)		WCB (cells/visual field)			BKA (%)				Roost category	Dietary niche
	Mean	SEM	Mean	SEM	*N*	Mean	SEM	ST	*N*		
Artibeus jamaicensis	56.38	2.53	11.59	2.24	13	62.31	6.52	69	11	2	Phytophagy
Artibeus lituratus	73.00	0.00	13.10	0.21	2	99.66	0.00	66	1	1.33	Phytophagy
Artibeus watsoni c.f.	12.21	0.41	4.60	1.18	7	54.83	7.91	70	8	1	Phytophagy
Carollia castanea	13.07	0.20	10.91	2.45	14	85.26	3.07	71	13	4.16	Phytophagy
Carollia perspicillata	18.14	0.58	9.89	1.95	11	82.74	5.29	68	12	3.91	Phytophagy
Carollia sowelli	16.75	0.58	9.14	2.05	10	94.58	3.34	62	11	4	Phytophagy
Desmodus rotundus	39.00	0.41	14.05	1.18	2	74.44	7.91	51	2	4.5	Carnivory
Ectophylla alba	5.40	0.06	1.11	0.36	13	86.56	5.32	59	11	1	Phytophagy
Glossophaga commissarisi	8.00	0.54	5.78	1.89	9	62.63	5.95	68	9	4.5	Phytophagy
Glossophaga soricina	7.82	0.30	3.92	1.44	5	62.57	7.62	62	4	4.66	Phytophagy
Lophostoma silvicolum	45.50	4.60	9.90	3.11	2	86.23	0.21	65	2	3	Insectivory
Mesophylla macconnelli	8.40	0.07	1.45	0.04	2	86.70	2.02	63	2	1	Phytophagy
Micronycteris hirsuta	14.50	0.00	3.90	0.00	1	96.11	0.00	70	1	3	Insectivory
Micronycteris microtis	7.00	0.71	1.80	0.42	2	75.49	11.56	67	2	3	Insectivory
Molossus currentium	18.83	0.96	2.52	0.44	6	94.46	2.10	52	5	3	Insectivory
Molossus sinaloe	26.50	0.00	2.50	0.00	1	43.19	0.00	50	1	4	Insectivory
Myotis elegans	5.30	0.00	2.20	0.00	1	86.77	0.00	65	1	2.5	Insectivory
Phyllostomus discolor	49.07	3.09	11.21	1.62	7	90.39	5.91	47	7	4	Phytophagy
Phyllostomus hastatus	121.13	8.31	14.53	4.40	8	91.68	6.67	65	5	2.6	Carnivory
Platyrrhinus helleri	14.67	1.21	3.63	1.04	3	93.48	1.41	57	3	1	Phytophagy
Rhynchonycteris naso	4.25	0.18	1.25	0.11	2	100.00	0.00	43	1	2	Insectivory
Saccopteryx bilineata	7.89	0.06	3.35	0.52	47	90.18	1.27	61	40	2.37	Insectivory
Saccopteryx leptura	5.20	0.14	0.60	0.07	2	87.58	5.25	63	2	3.5	Insectivory
Trachops cirrhosus	36.75	2.84	12.25	3.52	4	70.34	3.28	69	4	3.9	Carnivory

White blood cells have been counted on 10 visual fields under 200×magnification with a microscope on a monolayer smear. WBC gives the mean number of cells per visual field. Storage time (ST) of the plasma is given as mean days the samples have been stored at −80°C until assessment of BKA. Diet was drawn from La Val and Rodriguez [37], and species were assigned to three dietary niches: Carnivory (sanguinivorous vampire bat *Desmodus rotundus*, *Trachops cirrhosus* who is specialised on frogs, as well as *Phyllostomus hastatus*, who is omnivorous with a preference for vertebrates [60]), phytophagy (frugivorous and nectarivorous species) and insectivory. Roost category was drawn from Patterson and colleagues [26].

doi:10.1371/journal.pone.0054023.t001

Discussion

To our knowledge this is the first inter-specific comparative study investigating ecological factors associated with the immune system in free-ranging mammals. Our study demonstrates that the cellular immune system of bat species varies with diet and body mass, but is not associated with shelter choice, while the soluble part of the constitutive immune function increases with decreasing shelter permanence and protection, but does not vary with body mass and diet.

Based on total WBC counts, we found that the cellular immune system varied among bat species according to their trophic position. We found that total WBC count differed between dietary niches, with bat species preying at least partly on vertebrates or feeding on blood having the highest WBC counts. Carnivorous feeding habits may bear the highest putative risk of acquiring infectious diseases, as parasite and pathogen transmission is expected to be facilitated between more closely related species, i.e. bats and their vertebrate prey. Carnivorous bats apparently invest more in cellular immunity characterised by higher WBC counts in response to the potential threat of becoming infected with vertebrate-specific pathogens. While Nunn and colleagues [16] did not find a general association between carnivory and basal WBC counts, we argue that this might be due to the fact that Nunn and colleagues studied only healthy individuals in captive populations.

Phytophagous bats (frugivorous and nectarivorous species) had an intermediate number of WBCs. Although they do not prey on other vertebrate species, they still may face a notable risk of acquiring pathogens by ingesting contaminated plant matter [10,22,23]. Insectivorous bats had the lowest WBC counts. These bats catch prey by flying in open space such as above the canopy or between vegetation. Thus, close contact with potentially contaminated surfaces or infected food is either absent or minimal, and reports about pathogens that are potentially transmitted by ingesting insects are scarce.

Besides the correlation with dietary niche, we found that the cellular immune system of bats was influenced by body mass. This may either simply reflect an allometric relationship, or may have been caused by other factors associated with body mass. Nunn [15] found the number of neutrophils to increase with body mass in primates. He argued that larger primates are more terrestrial

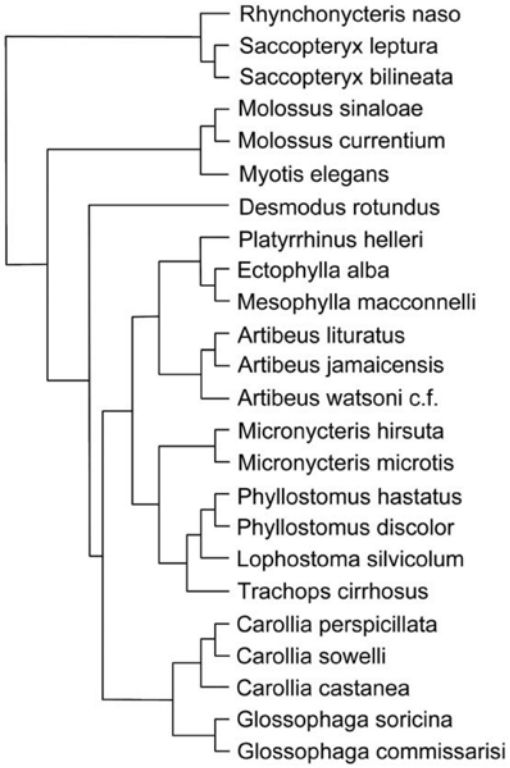


Figure 1. Phylogenetic tree of the 24 Neotropical bat species analysed, modified after Jones and colleagues [43]. As it is impossible to distinguish between *Artibeus watsoni* and *Artibeus phaeotis* in the field, we referred to it as *Artibeus watsoni c.f.* For statistical analysis, branch lengths were artificially computed as suggested by Grafen, with the length of a branch being the number of descending taxa minus 1 [45].
doi:10.1371/journal.pone.0054023.g001

than smaller ones, which may lead to body mass being a confounding rather than a causal factor when looking at the influence of ecological factors on the immune system. Vitone and colleagues [10] argued that larger animals also feed on more biomass, which increases the risk of ingesting infectious material. The amount of food ingested by bats can reach up to twice of their body mass (e.g. *Artibeus jamaicensis* [46]), thus large bats may have a higher risk of infestation by parasites and pathogens than small species. Another plausible explanation why large animals generally show a higher WBC counts may be that they are able to host a larger variety of pathogens ('host as island' hypothesis [47]). Thus, both diet and body mass are likely to contribute to the evolution of a species cellular immune system. However, there may also be additional factors influencing immunity that were not included in our study, such as physiological stress [48], reproductive status [49], season [50] or infection status.

We found different correlations between dietary niche, roost use and body mass and specific WBC types. While the number of lymphocytes increased with roost permanence and protection, the number of basophils decreased. Lymphocytes are the effectors of the adaptive immune system, having antigen specific cytotoxic (T cells) and secretory (B cells, producing antibodies) roles. Basophils on the other hand release histamines in certain immune responses and thus are important for allergic reactions. However, as the numbers of basophils are usually low (0–13.2% in bats, see ESM), correlations might be susceptible to outliers. Monocytes and eosinophils differed between dietary niches, with carnivorous bats having the highest number of cells, followed by phytophagous and insectivorous species. Monocytes and neutrophils constitute a first line immune defence against invading pathogens, eliminating the intruders via non-specific mechanisms, e.g. phagocytosis. Eosinophils destroy large parasites and are important for modulating allergic inflammatory responses. While the phagocytes respond quickly to a pathogen attack, more time is needed for selection and synthesis of the specific adaptive immune mediators. Thus, species living under higher and more diverse trophic-related risk of infection should rely on a quick non-specific immune defence, and may not invest as much in slower adaptive responses. However, slow-living species such as bats encountering repeated infections are thought to invest in adaptive immunity, while fast-living species should rely on less costly innate immunity due to their investment in growth and early reproduction ('pace-of-life' hypothesis [51]). Although white blood cell types have different, sometimes pathogen-specific functions, they are interlinked [51], which makes it difficult to attribute their levels to specific disease or pathogen risks. Thus, a detailed, causative interpretation of DWBC counts at this stage is difficult, but finding associations between DWBC count and ecological factors may potentially reflect the reaction of different cell types on niche-specific risks for pathogen infections.

The soluble part of the constitutive immune system assessed by BKA of the plasma decreased significantly with increasing shelter permanence and protection. A recent study on the bacterial killing ability of whole blood in the Brazilian free-tailed bat (*Tadarida brasiliensis*) [17] demonstrated that BKA may differ among individuals of a species that inhabit different roosting sites. Allen and colleagues argued that differences in ectoparasite abundances of roosts may be causative for differences in the innate immune system of *T. brasiliensis*. In Neotropical bats, ectoparasite load is largely influenced by the roosting behavior of bats: species roosting in more permanent and protected sites are more likely to be infected by parasitic flies and have an overall heavier parasite load [26]. Besides an increased abundance of ectoparasites, well protected shelters may also harbour more vectors for haemoparasites or may promote the accumulation of guano, which may be rich in various bacterial strains, viruses or parasites [52]. However, a higher ectoparasite prevalence found in more permanent shelters may also be caused by the low immunological protections of the animals roosting in such shelters. For example in humans, some pathogens are known to suppress both the cellular and humoral immune response [53]. Although the cause-effect relationship is difficult to assess for the case of bats, our result provides some evidence that the humoral immune system varies among species according to the used type of shelter. Alternatively, species roosting in more ephemeral sites may have to switch roosts more often than species roosting in more permanent sites. Theory predicts that more mobile species may encounter a higher parasite species richness. This theory has found support in mammals [54], birds [55] and fish [56]. For example, it has been found that migratory or dispersing bird species show stronger immune responses than non-migratory and non-dispersing species [57,58]. Thus, bat species roosting in more ephemeral sites may exhibit an increased BKA compared to species roosting in more permanent sites due to the necessity to change roosts more often and thus potentially coming into contact with more parasites and pathogens when exploring new environments.

Surprisingly, both ecological factors were only associated with one of the two measured aspects of the constitutive immune system, but not the other. Additionally, the effects differed between types of immune cells. Possibly, parasite and pathogen transmis-

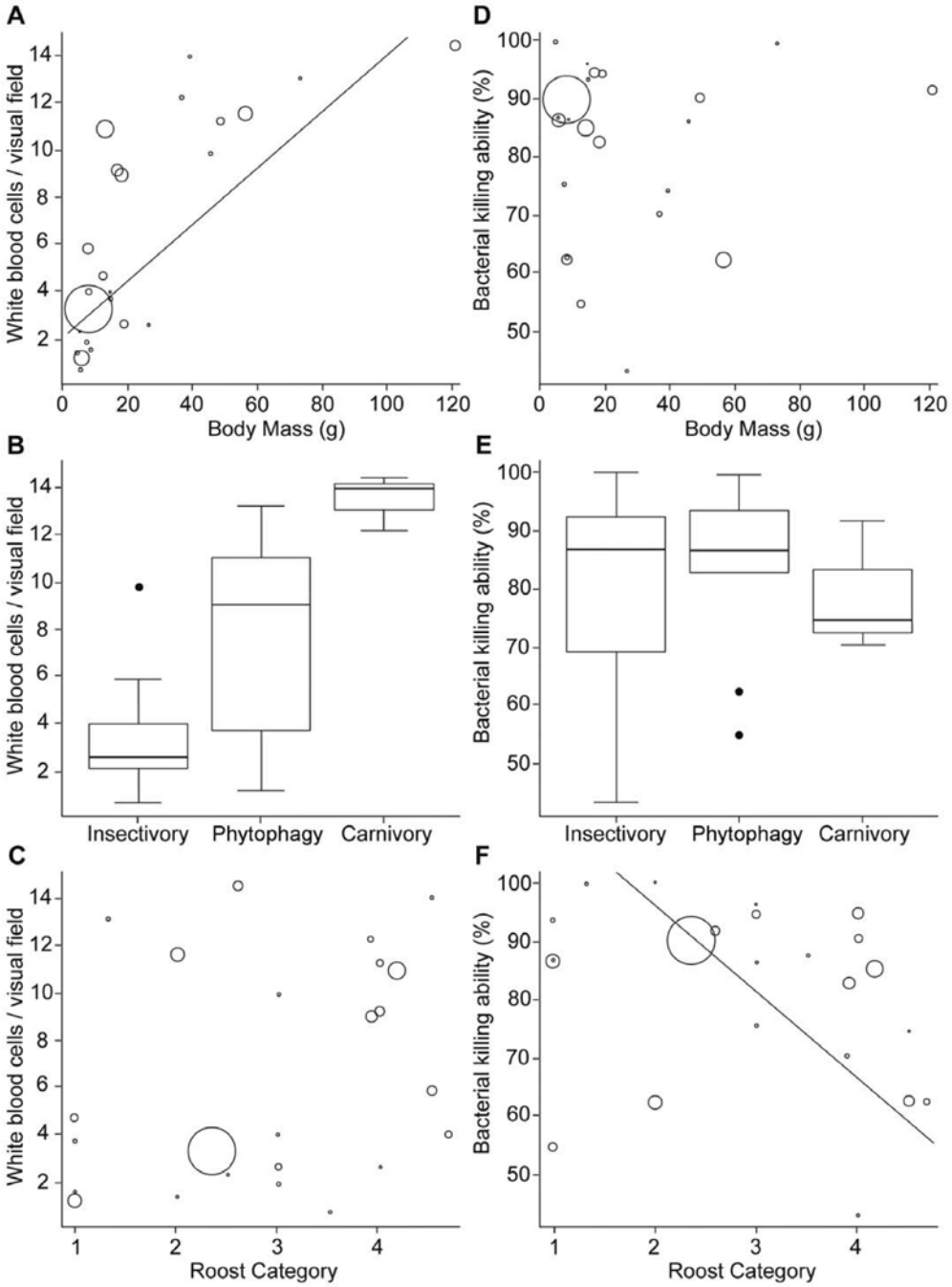


Figure 2. Association between ecological factors and different immune components. Sizes of circles indicate the sample size of each species. Body mass is positively associated with WBC counts, with large species having the highest number of white blood cells (A). WBC count varies among trophic levels (B): Bats feeding on blood and vertebrates (subsumed as carnivorous species) differ significantly in the number of WBCs from insectivorous species, while there is no significant difference between insectivorous and phytophagous bats and only a trend for a difference between phytophagous and carnivorous species. Roost category is not associated with WBC count (C). BKA is not associated with body mass (D). BKA does not differ between dietary niche (E) but decreases with increasing roost permanence and protection (F).
doi:10.1371/journal.pone.0054023.g002

sion risks vary between ecological factors, including those that might be interlinked with diet and shelter choice but could not be assessed in our study, for example group size, social system, species interactions or human influence. This may ultimately promote only certain domains of the immune system, e.g. cellular versus humoral immune system and adaptive versus innate immunity. While cellular immune functions are energetically costly to mount and maintain [59], humoral aspects are considered to be relatively inexpensive [12]. Thus, investment in different aspects of the immune system may be traded off against each other, depending

Table 2. Association between absolute numbers of different white blood cell types and ecological factors.

Lymphocytes (log-transformed)			
Body mass	**$F_{1,19}=13.2$**	**t = 3.6**	**p = 0.002**
Roost	**$F_{1,19}=8.0$**	**t = 2.8**	**p = 0.011**
Diet	$F_{2,19}=1.9$		p = 0.182
Monocytes			
Body mass	$F_{1,19}=0.03$	t = 0.17	p = 0.866
Roost	$F_{1,19}=4.2$	t = 2.0	p = 0.055
Diet	**$F_{2,19}=3.7$**		**p = 0.043**
Neutrophils			
Body mass	**$F_{1,19}=9.0$**	**t = 3.5**	**p = 0.008**
Roost	$F_{1,19}=1.3$	t = −1.8	p = 0.264
Diet	$F_{2,19}=2.0$		p = 0.159
Basophils			
Body mass	**$F_{1,19}=36.3$**	**t = 6.0**	**p<0.001**
Roost	**$F_{1,19}=6.0$**	**t = −2.4**	**p = 0.024**
Diet	$F_{2,19}=0.22$		p = 0.804
Eosinophils			
Body mass	$F_{1,19}=0.81$	t = 0.75	p = 0.381
Roost	$F_{1,19}=0.51$	t = −1.4	p = 0.485
Diet	**$F_{2,19}=5.5$**		**p = 0.013**

Sign of the t-value indicates direction of the correlation. P-values below $\alpha = 0.05$ are regarded as significant (in bold).
doi:10.1371/journal.pone.0054023.t002

on the resources an animal is able to invest in overall immunity. However, support for this notion has to come from experimental studies that focus on specific pathogens and the specific immune response of infected animal; a task that is difficult to approach under field conditions.

In conclusion, we provided first evidence from a comparative study that components of the immune system are associated with the ecological factors such as diet and roost use in free-ranging mammals. This implies that certain species are more prone to acquire infectious diseases due to their trophic position or the selection of shelter. Such insights on the effect of ecological factors on immunity and putative disease risk are not only important for conservation, but also to understand potential disease transmission risk and disease dynamics in bats and other mammals.

Supporting Information

Table S1 Differential white blood cell counts (absolute and relative mean as well as SEM) of the 24 Neotropical bat species.
(DOC)

Acknowledgments

We are grateful to Ivailo M. Borissov and Silke L. Voigt-Heucke for help in the field, to Katja Pohle for her help during laboratory analyses and Alexandre Courtiol for statistical advice. We thank the Organization for Tropical Studies (OTS) for allowance to use their facilities and for providing logistic support and the Costa Rican authorities for granting permission to conduct research.

Author Contributions

Fieldwork: KS CCV. Laboratory work: KS GAC. Supervision: CCV. Conceived and designed the experiments: KS GAC CCV. Performed the experiments: KS GAC CCV. Analyzed the data: KS. Wrote the paper: KS GAC CCV.

References

1. Lehmann T (1993) Ectoparasites: direct impact on host fitness. Parasitol Today 9: 8–13.
2. Alexander RD (1974) The evolution of social behavior. Annu Rev Ecol Syst 5: 325–383.
3. Altizer S, Nunn CL, Thrall PH, Gittleman JL, Antonovics J, et al. (2003) Social organization and parasite risk in mammals: integrating theory and empirical studies. Annu Rev Ecol Evol Syst 34: 517–547.
4. Møller AP, Dufva R, Allander K (1993) Parasites and the evolution of host social behavior. Adv Study Behav 22: 65–102.
5. Brown CR, Komar N, Quick SB, Sethi RA, Panella NA, et al. (2001) Arbovirus infection increases with group size. Proc R Soc Lond B 268: 1833–1840.
6. Côté IM, Poulin R (1995) Parasitism and group size in social animals: a meta-analysis. Behav Ecol 6: 159–165.
7. Czirják GÁ, Møller AP, Mousseau TA, Heeb P (2010) Microorganisms associated with feathers of barn swallows in radioactively contaminated areas around Chernobyl. Microb Ecol 60: 373–380.
8. Møller AP, Czirják GÁ, Heeb P (2009) Feather micro-organisms and uropygial antimicrobial defences in a colonial passerine bird. Funct Ecol 23: 1097–1102.
9. Bell G, Burt A (1991) The comparative biology of parasite species diversity: internal helminths of freshwater fish. J Anim Ecol 60: 1047–1064.
10. Vitone ND, Altizer S, Nunn CL (2004) Body size, diet and sociality influence the species richness of parasitic worms in anthropoid primates. Evol Ecol Res 6: 183–199.
11. Saino N, Bolzern AM, Møller AP (1997) Immunocompetence, ornamentation, and viability of male barn swallows (*Hirundo rustica*). Proc Natl Acad Sci USA 94: 549–552.
12. Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88: 87–98.
13. Stearns SC (1992) The evolution of life histories. Oxford University Press Oxford.
14. Nunn CL, Gittleman JL, Antonovics J (2000) Promiscuity and the primate immune system. Science 290: 1168–1170.
15. Nunn CL (2002) A comparative study of leukocyte counts and disease risk in primates. Evolution 56: 177–190.
16. Nunn CL, Gittleman JL, Antonovics J (2003) A comparative study of white blood cell counts and disease risk in carnivores. Proc R Soc Lond B 270: 347–356.
17. Allen LC, Turmelle AS, Mendoca MT, Navara KJ, Kunz TH, et al. (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.
18. Zysling DA, Garst AD, Demas GE (2009) Photoperiod and food restriction differentially affect reproductive and immune responses in Siberian hamsters *Phodopus sungorus*. Funct Ecol 23: 979–988.
19. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T (2006) Bats: important reservoir hosts of emerging viruses. Clin Microbiol Rev 19: 531–545.
20. Wibbelt G, Moore MS, Schountz T, Voigt CC (2010) Emerging diseases in Chiroptera: why bats? Biol Lett 6: 438–440.
21. Lorch JM, Meteyer CU, Behr MJ, Boyles JG, Cryan PM, et al. (2011) Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. Nature 480: 376–378.
22. Chua KB, Lek Koh C, Hooi PS, Wee KF, Khong JH, et al. (2002) Isolation of Nipah virus from Malaysian island flying-foxes. Microb Infect 4: 145–151.
23. Fogarty R, Halpin K, Hyatt AD, Daszak P, Mungall BA (2008) Henipavirus susceptibility to environmental variables. Virus Res 132: 140–144.
24. Kuzmin IV, Bozick B, Guagliardo SA, Kunkel R, Shak JR, et al. (2011) Bats, emerging infectious diseases, and the rabies paradigm revisited. Emerg Health Threat J 4: 7159.
25. Kunz TH, Lumsden LF (2006) Ecology of cavity and foliage roosting bats. In: Kunz TH, Fenton MB, editors. Bat ecology. University of Chicago Press.
26. Patterson BD, Dick CW, Dittmar K (2007) Roosting habits of bats affect their parasitism by bat flies (Diptera: Streblidae). J Trop Ecol 23: 177–189.
27. Moore MS (2011) Ecological immunology in little brown myotis (*Myotis lucifugus*; Chiroptera) affected by white-nose syndrome [dissertation]. Boston University.
28. Walberg J (2001) White blood cell counting techniques in birds. Semin Avian Exot Pet 10: 72–76.
29. Timm RM, LaVal RK (1998) A field key to the bats of Costa Rica. Occasional Publication Series, University of Kansas Center of Latin American Studies 22: 1–30.

30. Schinnerl M, Aydinonat D, Schwarzenberger F, Voigt CC (2011) Hematological survey of common neotropical bat species from Costa Rica. J Zoo Wildl Med 42: 382–391.
31. Durbin C, Guo K, Hoffman W, Eric Schultze A, White S (2009) Estimating leukocyte, platelet, and erythrocyte counts in rats by blood smear examination. Vet Clin Path 38: 157–162.
32. Tvedten H, Grabski S, Frame L (1988) Estimating platelets and leukocytes on canine blood smears. Vet Clin Path 17: 4–6.
33. Ezenwa VO, Ekernas SL, Creel S (2012) Unravelling complex associations between testosterone and parasite infection in the wild. Funct Ecol 26: 123–133.
34. Tieleman BI, Williams JB, Ricklefs RE, Klasing KC (2005) Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. Proc R Soc Lond B 272: 1715–1720.
35. Moore MS, Reichard JD, Murtha TD, Zahedi B, Fallier RM, et al. (2011) Specific alterations in complement protein activity of little brown myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. PLoS One 6: e27430.
36. Liebl AL, Martin LB (2009) Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry. Funct Ecol 23: 1091–1096.
37. LaVal RK, Rodríguez B (2002) Murcielagos de Costa Rica. Editorial INBio.
38. R Development Core Team (2010) R: A language and environment for statistical computing, version 01/19. R foundation for statistical computing. http://www.r-project.org.
39. Price T (1997) Correlated evolution and independent contrasts. Philos Trans R Soc London B 352: 519–529.
40. Pagel M (1999) Inferring the historical patterns of biological evolution. Nature 401: 877–884.
41. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2009) Nlme: linear and nonlinear mixed effects models. R package version 3.1–96, version.
42. Paradis E, Claude J, Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20: 289–290.
43. Jones KE, Purvis A, Maclarnon ANN, Bininda-Emonds ORP, Simmons NB (2002) A phylogenetic supertree of the bats (Mammalia: Chiroptera). Biol Rev Camb Philos Soc 77: 223–259.
44. Hoffmann FG, Baker RJ (2003) Comparative phylogeography of short-tailed bats (Carollia: Phyllostomidae). Mol Ecol 12: 3403–3414.
45. Grafen A (1989) The phylogenetic regression. Philos Trans R Soc London B 326: 119–157.
46. Morrison DW (1978) Foraging ecology and energetics of the frugivorous bat *Artibeus jamaicensis*. Ecology 59: 716–723.
47. Kuris AM, Blaustein AR, Alio JJ (1980) Hosts as islands. Am Nat 116: 570–586.
48. Davis AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol 22: 760–772.
49. Cichon M (2001) Diversity of age-specific reproductive rates may result from ageing and optimal resource allocation. J Evol Biol 14: 180–185.
50. Buehler DM, Piersma T, Matson K, Tieleman BI (2008) Seasonal redistribution of immune function in a migrant shorebird: annual-cycle effects override adjustments to thermal regime. Am Nat 172: 783–796.
51. Previtali MA, Ostfeld RS, Keesing F, Jolles AE, Hanselmann R, et al. (2012) Relationship between pace of life and immune responses in wild rodents. Oikos 121: 1483–1492.
52. Li L, Victoria JG, Wang C, Jones M, Fellers GM, et al. (2010) Bat guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. J Virol 84: 6955–6965.
53. Greenwood BM, Whittle HC, Molyneux DH (1973) Immunosuppression in Gambian trypanosomiasis. Trans R Soc Trop Med Hyg 67: 846–850.
54. Bordes F, Morand S, Kelt DA, Van Vuren DH (2009) Home range and parasite diversity in mammals. Am Nat 173: 467–474.
55. Pérez-Tris J, Bensch S (2005) Dispersal increases local transmission of avian malarial parasites. Ecol Lett 8: 838–845.
56. Bell G, Burt A (1991) The comparative biology of parasite species diversity - internal helminths of freshwater fish. J Anim Ecol 60: 1047–1063.
57. Moller AP, Martín-Vivaldi M, Soler JJ (2004) Parasitism, host immune defence and dispersal. J Evol Biol 17: 603–612.
58. Moller AP, Erritzoe J (1998) Host immune defence and migration in birds. Evol Ecol 12: 945–953.
59. Newsholme P, Newsholme EA (1989) Rates of utilization of glucose, glutamine and oleate and formation of end-products by mouse peritoneal macrophages in culture. Biochem J 261: 211–218.
60. Santos M, Aguirre LF, Vázquez LB, Ortega J (2003) Phyllostomus hastatus. Mamm Species 722: 1–6.

Chapter II

Immunological parameters are linked to age and survival in a long-lived mammal, the greater sac-winged bat (*Saccopteryx bilineata*)

(Submitted)

Immunological parameters are linked to age and survival in a long-lived mammal, the greater sac-winged bat (*Saccopteryx bilineata*)

Karin Schneeberger, Gábor Á. Czirják, Christian C. Voigt

Abstract

Despite its central importance to fight pathogens, the immune system imposes costs that may have to be traded against life-history traits such as longevity. Yet, it is unknown if a trade-off with longevity occurs in free-ranging mammals. Here, we asked if age and survival are connected to immune parameters in the greater sac-winged bat *Saccopteryx bilineata*. We measured total white blood cell (WBC) counts, bacterial killing ability (BKA) of the plasma and immunoglobulin G (IgG) concentration, and assessed the age and survival of each individual based on our long-term population study. We found individuals with high WBC counts to disappear from the population during subsequent years. In agreement with this observation, immune cell numbers decreased with increasing age of cohorts. Similarly, although not connected with age, individuals were no longer encountered in the colonies half a year after relatively high BKA were measured. IgG concentrations increased with age but were not connected with disappearance. Our study provides evidence for age-related changes in immunological parameters in a free-ranging mammal population and reveals that elevated levels of immune components may be associated with an increased mortality risk.

Keywords: Immune system, age, bats, eco-immunology, survival

Introduction

By exploiting host resources, parasites and pathogens inflict damages and therefore impose a negative effect on the host fitness (Lehmann 1993). Removal, repelling or avoidance of parasites or pathogens are examples of behavioural adaptations by the host to prevent costs associated with infections. Next to behavioural defence strategies, the immune system provides an animal with a set of cellular, biochemical and molecular defence mechanisms against potentially harmful agents. However, mounting and maintaining an immune response is energetically costly (Lochmiller and Deerenberg 2000), leading to a potential trade-off with other life-history traits. For example, it has been shown that the immune system may get impaired when energy has to be allocated to reproduction (Christe et al. 2000; Christe et al. 2007). It is therefore assumed that animals are vulnerable to diseases because prioritising the maintenance of an overall high level of immunity would besides other negative consequences compete with the need for investment in other important life-history traits (Zuk and Stoehr 2002).

For example, immune parameters may be connected with ageing and survival. In fact, survival and thus longevity are central fitness parameters for animals with a slow pace of life, because prolonged survival may increase lifetime breeding success. Studies on the relation between immune response and survival are scarce and – as with many eco-immunological research topics – concentrate on birds. Eraud and colleagues (2009) for example mimicked bacterial infections in nestlings of Eurasian collared dove (*Streptopelia decaocto*) by injecting antigens from *Escherichia coli*. The authors found that antigen treated nestlings were more likely to become victim of a predator than control birds. However, in male barn swallows (*Hirundo rustica*), it has been shown that individuals that produce higher titres of antibodies against sheep red blood cells are more likely to survive until the next breeding season compared to birds that produced lower titres (Saino et al. 1997). In contrast, breeding female eider ducks (*Somateria mollissima*) that mounted a humoral immune response against three agents were less likely to return than females that did not mount an immune response (Hanssen et al. 2004). Only little attention has been paid to a possible trade-off between immune function and survival in mammals, particularly in free-ranging populations. For laboratory model organisms such as mice (*Mus mus domesticus*) for example, it is known that inhibition of anti-inflammatory cytokines reduces longevity (Belloni et al. 2010). However, studies on laboratory species may mask the effect of natural selection, which is why studies on free-ranging mammals are urgently needed to shed light on the functional relationship between the immune

system and survival (Ricklefs and Wikelski 2002). To our knowledge, there is only a single study on the link between selected immune components and fitness in a wild mammal. A study on free-ranging Soay sheep (*Ovis aries*) found that antinuclear antibody concentrations are connected with reduced reproduction but increased survival during harsh winters (Graham et al. 2010). Yet, it is thus far unclear how other aspects of the immune system are associated with survival and longevity in mammals.

With increasing age, irreversible physiological and molecular changes accumulate and impair the performance of individuals, including the immune system (Monaghan et al. 2008). Evidence for a decrease in immune functions with age has been found in humans, domestic animals and laboratory rodents (Utsuyama et al. 1992; Ginaldi et al. 2001; Blount et al. 2005; Frasca et al. 2005; Kondratov et al. 2006; Linton et al. 2006; Noreen et al. 2011), as well as in Soay sheep (Nussey et al. 2012). The link between the immune system, age and survival may be especially interesting in Chiroptera, since the maximal recorded life span of bats is on average 3.5 times higher than those of similar sized terrestrial species (Austad and Fischer 1991; Brunet-Rossinni and Austad 2004; Nussey et al. 2012). The immune competence of bats is also drawing increasing attention because bats host some of the most prominent zoonotic viral pathogens (Wibbelt et al. 2010; Wood et al. 2012), such as lyssaviruses (Kuzmin et al. 2011), paromyxoviruses (e.g. Hendra, Nipah; Drexler et al. 2012) and filoviruses (e.g. Ebola and Marburg; Monath 1999); yet bats sometimes do not show any signs of suffering or sickness. In contrast, North American bats suffer heavily from the newly emerging infectious disease known as white-nose syndrome (Lorch et al. 2011), probably as a consequence of immune deficiency (Moore 2011; Moore et al. 2011). Therefore, a better knowledge of the bat immune system might help to explain why bats vary largely in their susceptibility towards pathogens. Recent studies show that the number of immune cells varies greatly among species (Schinnerl et al. 2011), with carnivorous bats having especially high numbers of immune cells (Schneeberger et al. 2013). Small frugivorous bats on the other hand have some of the lowest white blood cell counts ever recorded in mammals. The humoral immune system is also variable depending on the potential pathogen transmission risk in roosts (Schneeberger et al. 2013), with species living in more permanent and protected roosts showing higher humoral immune activity than species roosting in ephemeral sites. Within species, immune responses are known to vary between colonies and types of roosts (Allen et al. 2009).

Here, we asked if measures of the cellular and humoral immune system are linked to individual survival in a free-ranging population of greater sac-winged bats (*Saccopteryx*

bilineata). Furthermore, we tested if age affects measures of the cellular and humoral immune system. We made use of data collected during long-term studies of this species: As part of this long-term study, all members of a colony were identifiable based on coloured rings that were attached to the forearm of bats a few weeks after birth. We monitored the presence or absence of colony members twice each year. Once adults have settled in a colony at an age of about 6 months, greater sac-winged bats exhibit a high roost fidelity (Voigt et al. 2007; Voigt and Schwarzenberger 2008). Therefore, disappearance of adult bats from a colony is most likely caused by death. Differences in immunocompetence between sexes are a common phenomenon among vertebrates, with males showing often inferior immune functions compared to females (Folstad and Karter 1992). We therefore investigated additionally if a possible link between immune parameters and age or survival is sex-specific. Furthermore, we included body condition as an additional factor in our study, since individuals in better condition may be able to invest in both immunity and longevity to a larger extent than individuals in bad condition (Sheldon and Verhulst 1996). We measured aspects of the cellular immunity (total white blood cell (WBC) counts) as well as aspects of the humoral immune system (the adaptive immune system quantified by assessing immunoglobulin G concentration; IgG, and aspects of the innate immune function being measured by the bacterial killing ability of the plasma; BKA) in bats of known age. We predicted that variation in immunity influences mortality and that the selected immunological parameters vary with sex and age in *S. bilineata*.

Materials and methods

Greater sac-winged bats show a high roost fidelity and are therefore an ideal study system for age-related questions. At our Costa Rican study site, this species has been studied for more than 15 years. Six colonies roost in abandoned buildings surrounded by primary and secondary rain forest at “La Selva” Biological Station (10°25’N; 84°00’W, Province Heredia). As part of a long-term project all individuals are marked with numbered and coloured plastic rings (AC Hughes LTD., Middlesex, UK, size XCL) and the date of the first capture is reported in a database. Most juveniles were caught and marked during their year of birth in summer, so that we know the age of all individuals that were marked as juveniles. However, new unmarked adult individuals are occasionally caught, of which we do not have direct information about the year of birth. The age of these individuals was therefore estimated with the help of the knowledge we have on the social structure of this species: We assumed that unmarked females found in colonies after the lactation period are

immigrants that were born either in the year of capture if caught in winter (age = 1) or in the previous year (age = 2) if caught in summer. This is based on the finding that females disperse after weaning in late summer to avoid inbreeding with their father, while male offspring remain in their natal colony (Nagy et al. 2007). We assigned the age of males that were marked as adults in the same way, assuming that unmarked adult males were not caught during their juvenile stage, as they were probably roosting in a more remote place of the colony. Such assignments were done for in total 26 individuals covering all age classes.

Estimating survival

In contrast to most other bats, *S. bilineata* clings to vertical surfaces rather than horizontal ones. In our study population, each individual is marked with a unique combination of ring colours and individuals do not cluster but remain minimum distances of 5-8 cm from each other (Bradbury and Emmons 1974). Furthermore, the colonies are well habituated to the presence of an observer, which is why it is possible to identify each bat from a distance of 3-8 meters. Census was done biannually during the day either by taking photographs of the colony and identifying the individuals later on by matching the ring colours with the database, or by writing down the colour of the rings observed with binoculars. In order to estimate survival, we checked whether an individual was reported to be present in the colony 6-8 months after the sampling. The probability for a male being present in the colony on a particular day has previously been found to be 98% (Voigt et al. 2007), while females once they immigrated in a colony had a mean probability of 91% (median 98%) to be present (Voigt and Schwarzenberger 2008). We visited each colony at least twice during each semester. If an individual was not encountered during both visits and in the subsequent semesters, we assumed it being permanently disappeared from the colony after the year of sampling. At one roosting site, human induced disturbance led to the disappearance of the whole colony. We therefore excluded these individuals (N=12) from the analysis on the effect of immune parameters on survival, as disappearance does not necessarily account for mortality.

Sample collection

Blood samples were collected in November and December 2010 and 2011. We captured bats when they returned to their roost at dusk between 5:15 and 6:15 am using monofilament nylon mist nets (2.5 m height, 3 or 6 m length, Ecotone, Gdynia, Poland)

placed at a minimum distance of 2 m from their daytime roosts. In one roost, bats were captured with hand nets. As the capturing took place outside the reproductive season, none of the females were pregnant or lactating. We identified the sex of all bats and weighed all individuals using an electronic balance (accuracy at 0.1 g) and forearm length was measured using a calliper. We calculated body condition index (BCI) as body mass/forearm, a standard method to measure energy reserves relative to a structural element of the body in bats (Pearce et al. 2008; Reynolds et al. 2009). We took approximately 50 µl of blood from a total of 79 clinically healthy individuals (55 in 2010 and 24 in 2011) by punctuating the antebrachial vein with a sterile needle and collecting the blood with sterile heparinised capillaries. One drop was used for preparing blood smears on glass slides (Microscope Slides (76x26mm), cut edges, Menzel, 38116 Braunschweig, Germany). Of 64 individuals, we gained enough blood to extract the plasma after centrifugation at 11,500 rpm for 10 min. Plasma was stored at -80°C in two aliquots, one for BKA measurement, one for quantification of IgGs. After sampling, bats were immediately released at the site of capture.

Capturing and sampling of the bats was approved by the Internal Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research (Approval No. 2010-09-01) and by the local authorities of Costa Rica (No. 137-2010-SINAC and 168-2011-SINAC).

White blood cell counts

Blood smears were stained with May-Gruenwald's solution (#T863.2, Carl Roth GmbH) and Giemsa (#T862.1, Carl Roth GmbH). We manually estimated total white blood cell (WBC) count per microliter by counting 10 visual fields with a microscope under 200 x magnification, as has been done in bats before when blood sample volumes were limited (Moore 2011; Schneeberger et al. 2013). In order to increase the sample size, we additionally analysed blood smears of individuals sampled from the same population in 2009 (Schinnerl et al. 2011). As in previous studies on vertebrate immune systems, we assumed that the number of WBCs represents a reasonable measure of investment in cellular immunity (Nunn et al. 2000; Nunn 2002; Nunn et al. 2003).

Bacterial killing ability

Constitutive innate immune function was measured by assessing the bacterial killing activity of the plasma against *Escherichia coli* (Tieleman et al. 2005), an assay that has

been used on bats before (Allen et al. 2009; Schneeberger et al. 2013). The individual plasma samples were diluted 1:20 with a CO_2-independent media (#18045, Gibco-Invitrogen, CA) enriched with 4mM L-Glutamine (#25030, Gibco-Invitrogen, CA) and 5% Fetal Calf Serum (#S0115, Biochrom AG). Ten µl of a suspension of living *Escherichia coli* (ATCC #8739) was added to 140 µl of diluted sample. Previously, the number of bacteria in the suspension was adjusted in order that 50 µl of plasma-bacteria mixtures produces approximately 200 colonies.

The plasma-bacteria mixtures were then incubated for 30 min at 37ºC and afterwards, 50 µl aliquots were spread onto Tryptic Soy Agar plates (#CP70.1, Carl Roth GmbH) in duplicates. To obtain the number of bacteria that we had before the interaction with the plasma, we diluted the bacterial suspension with 140 µl media without plasma and plated the mixture without previous incubation. We incubated the plates overnight at 37°C and counted the number of colonies formed the following day. The bacterial killing activity was calculated as 1 – (average of the viable bacteria after incubation / the initial number of bacteria) and the average was taken from two plates per sample (Schneeberger et al. 2013).

Immunoglobulin G concentration

Immunoglobulin G (IgG) levels were quantified using a Protein G ELISA (Ross et al. 1993; Stöbel et al. 2002). Protein G, a streptococcal protein, binds IgG from a number of different wildlife species (Stöbel et al. 2002), and it has been validated to be used in routine serological testing of different bat species for specific antibody levels (Wellehan et al. 2009). Following initial checkerboard titrations, we coated microtiter plates (Nunc-ImmunoTM Plate, NUNCTM Brand Products) with 100 µl of diluted plasma sample (diluted 1:10.000 in 50 mM NaHCO3, pH 9,5) and incubated for 1 hour at 37°C. After incubation, plates were washed twice with Tris-buffered saline-Tween20 solution (TBS-Tween20). Two hundred µl of 1% gelatine solution (#104070, Merck) was added to each well for blocking non-specific reagent binding and plates were incubated for 30 minutes at 37°C. Subsequently, plates were washed as described above and 100 µl of Protein G–horseradish peroxidase conjugate (#P-21041, Invitrogen) at a dilution of 1:12.000 in TBS-Tween20 solution was added to each well. Following 30-minute incubation at room temperature, the plates were washed five times in TBS-Tween20. Wells were then submerged with 100 µl of freshly prepared TMB solution [10% 3,3',5,5'-tetramethylbenzidine (#0411-01, SouthernBiotech) dissolved in DMSO (#D5879, Sigma Aldrich) was diluted 1:100 in phosphate-citric-buffer and mixed with 30% H_2O_2

(#7475456, Hedinger)] and the reaction was stopped with 1M acid sulphuric after eight minutes. Plates were then immediately transferred to a microplate reader (Biotek) and the absorption was read at 450 nm. According to the Beer–Lambert law, antibody concentration is directly proportional to the absorption. We therefore conducted statistical analysis on the mean optical density (OD) of duplicates measured by the microplate reader for each sample.

Statistical Analysis

We used two-sampled t-tests to test for general differences between males and females in body measurements and Wilcoxon rank sum tests where data were not normally distributed. We calculated mixed effects models using age (in years), sex and BCI as fixed factors and immune parameters as response variable. Likelihood ratio tests for nested models were performed to choose the model that explains the variation in immune measures the best. We added each factor (age, sex, BCI) one by one to the model and compared it with the previous model not including the additional factor. Factors included in the last significantly different model were then analysed separately for their effect on immune parameters. To look at whether age has a sex-specific effect on immune parameters, we included a model with interaction between sex and age and compared it to models without interaction when both factors were significant. Individual identity was included as random factor in all models, as some individuals were measured twice in different years, and thus contribute to two age classes. As data were not normally distributed but followed a poisson distribution, all test employed a poisson correction. Due to low sample sizes at age 4 (N=6), 5 (N=7) and 6 (N=3) as compared to 1 (N=32), 2 (N=39) and 3 (N=16), we additionally performed the same analysis with ages from 4 to 6 being grouped (“4+”; N=16).

To test whether variation in immune parameters predict presence or absence of individuals in the colony half a year after sampling, we calculated residuals as the difference between individual datapoints and the age-specific estimate for an immune parameter. For statistical analysis, we separated individuals of one year of age from other age classes, as during the first year, females usually disperse, which is why disappearance of these individuals from the colony may not account for mortality. Individuals aged 2-6 years were grouped, as these animals do not disperse and thus absence from the colonies most likely indicates death of the individual. We conducted Wilcoxon rank sum tests for

differences between individuals that were present in the colony and individuals that were absent in the subsequent semesters.

All tests were performed with R statistical software (version 2.13.1; R R Development Core Team 2010) using the package "lme4" for the mixed effect models. Parameters are presented in the results section as means ± one standard deviation.

Results

In total, we captured 79 individuals from 5 colonies at our study site in Costa Rica (25 males and 30 females in 2010, 7 males and 17 females in 2011) and used blood smears of additional 24 individuals (11 males and 13 females) captured in 2009. Males and females differed significantly in body mass (t=-2.303; df=85.18; p=0.024) and forearm length (W=2126; p<0.001), with females being larger (forearm length: females: 46.88 ± 1.39 mm; males: 44.96 ± 1.13 mm) and heavier than males (body mass: females: 8.18 ± 0.54 g; males: 7.93 ± 0.53 g). BCI did not differ between the sexes (t=0.751; df=88.09; p=0.455) and did not change with age (X^2=0.004; p=0.949). Age did not have an influence on whether an individual was still present or absent half a year after sampling (W=954.5; p=0.521). BCI was not related to the disappearance of neither animals in their first year (t=-0.654; df=28.51; p=0.518) nor older individuals (t=0.034; df=57.00; p=0.973).

Association of immune parameters with age

WBC counts of *S. bilineata* were best explained by a model including all three factors sex, age and BCI (X^2=5.379; p=0.020). WBC counts significantly decreased with age (X^2=-2.876; p=0.004; Fig. 2a) and differed at p = 5% between the sexes (X^2=3.827; p=0.050), with males tending to have higher numbers of WBC than females (males: 2.56 ± 2.01 cells per visual field; N=38; females: 2.20 ± 3.50 cells per visual field; N=54). There was no interaction between sex and age (X^2=0.169; p=0.681), indicating that age affected WBC counts in both males and females to the same extent. WBC counts were positively associated with BCI (X^2=5.377; p=0.020). However, when correcting for BCI, WBC counts still decreased with age (X^2=9.140; p=0.003) and tended to be higher in males than females (X^2=3.240; p=0.072). With ages from 4 to 6 being grouped to one age class, WBC counts still significantly decreased with age (X^2=9.360; p=0.002), and the effect was even stronger when correcting for BCI (X^2=10.359; p=0.001).

Variations in BKA was best explained by sex only (X^2=2.997; p=0.083), with males tending to have higher BKA than females (males: 87.51 ± 10.87 %; N=24; females: 80.44

± 20.29 %; N=40). Age had no significant influence on BKA (X^2=0.002; p=0.964; Fig. 2b), even with ages from 4 to 6 being grouped in one age class (X^2=0.011; p=0.917). BCI was not associated with BKA (X^2=1.648; p=0.199).

IgG OD increased significantly with age (X^2=2.070; p=0.039; Fig. 2c), with a stronger effect when grouping ages from 4 to 6 (X^2=4.754; p=0.029). IgG OD did not differ between males and females (X^2=0.034; p=0.854; Fig. 2c) and was not correlated with BCI (X^2=0.351; p=0.554).

Immune parameters as predictor for presence/absence of individuals

In individuals of one year age, 14 out of 27 individuals (~50%) disappeared from the colonies half a year after blood collection. The likelihood for disappearing from colonies for older individuals was: 18 out of 33 aged 2 years, 4 out of 12 aged 3 years, 4 out of 6 aged 4 years, 2 out of 5 aged 5 years and 1 out of 2 aged 6 years. In total, 50% of all individuals older than one years of age were not observed the year after sampling.

When controlling for age, individuals in their first year that were still present in the colony did not differ in WBC counts (W=39; p=0.661; Fig 1a), BKA (W=29; p=0.864; Fig 1b) or IgG OD (W=13; p=0.354; Fig 1c) from individuals that were absent at the time of census half a year later. In older age classes, individuals that were present in the colonies had significantly lower residual WBC counts than individuals that were not present (W=276.5; p=0.002; Fig. 1a). Similarly, residual BKA was higher in present individuals compared to individuals that were absent (W=111.5; p=0.005; Fig. 1b). Residual IgG OD did not differ between present and absent individuals (W=173; p=0.130; Fig. 1c).

Fig. 1 (next page): Effect of age on measures of the immune system in *S. bilineata*. Dots indicate individual measures while the solid line outlines the expected values deriving from the model. White blood cell (WBC) count decreased with increasing age (a), while bacterial killing ability (BKA) of the plasma did not change with age (b). Concentration of immunoglobulins (IgG) (given as the mean optical density (OD) of the protein G ELISA) increased significantly with age (c).

Fig. 2 (next page): Differences in measures of the immune system between individuals present and absent from the roost half a year after sampling. White boxes are individuals at one year of age, grey boxes individuals older than one year. White blood cell (WBC) count (a) and BKA (b) did not differ between present and absent individuals of the first year of age, but was lower in older individuals that were still present in the colony compared to individuals that disappeared. Concentration of immunoglobulins (IgG; given as the mean optical density (OD) of the protein G

ELISA; c) did not differ between present and absent individuals, neither in young animals nor in animals ranging from age 2 to 6 years.

Fig. 1

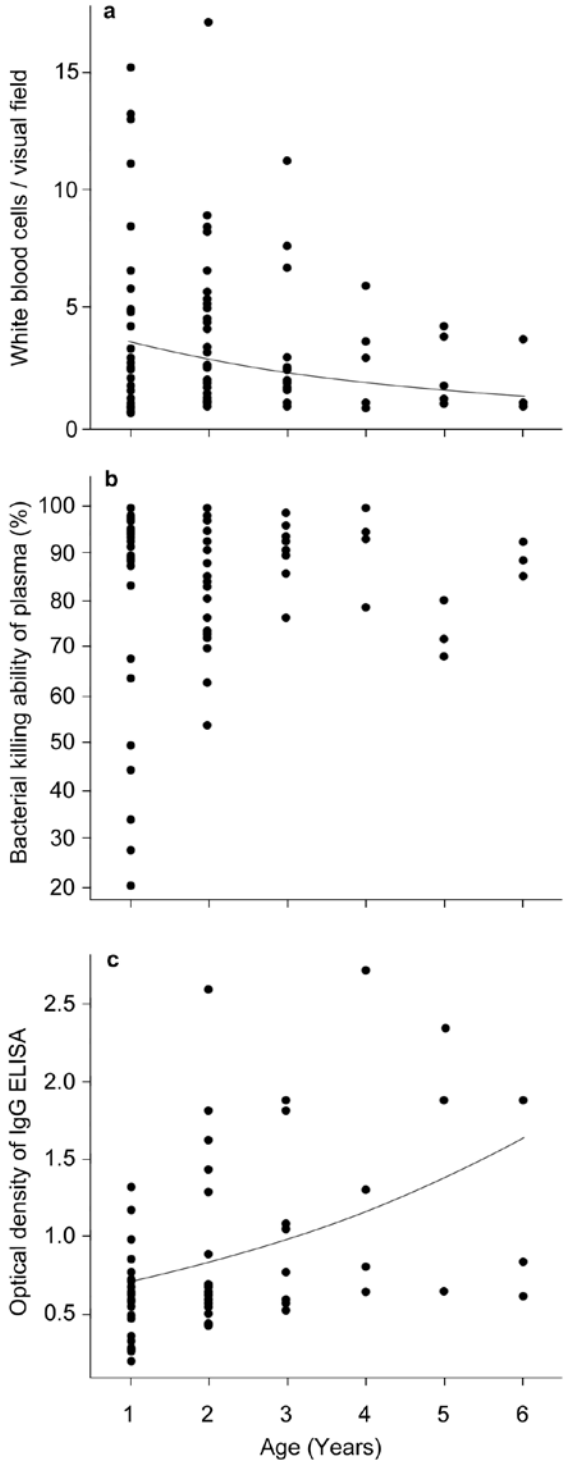
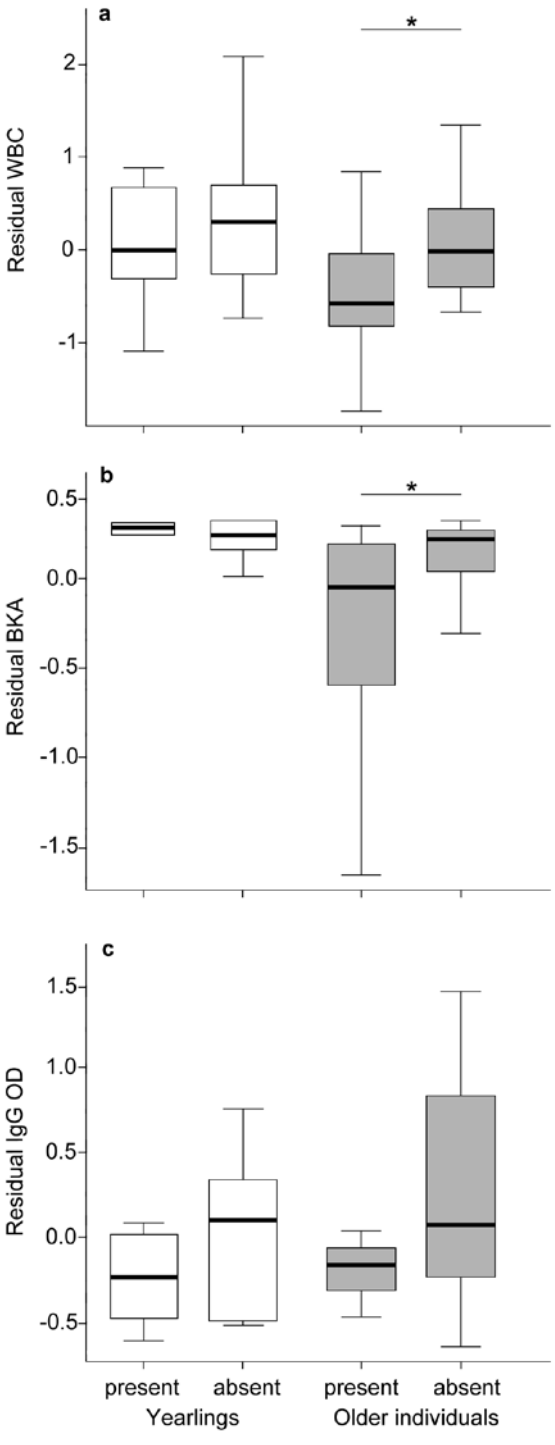


Fig. 2



Discussion

We obtained a dataset on several aspects of the immune system of greater sac-winged bats (*Saccopteryx bilineata*) ranging from 1 to 6 years of age; the average lifespan of *S. bilineata* (Wilkinson and South 2002). We found an effect of both sex and age on selected immune parameters. Furthermore, we found that measurements of the cellular and humoral immune system potentially predict an individuals' survival in the colony.

In general, male greater sac-winged bats tended to have a higher number of immune cells and bacterial killing ability (BKA) of plasma than females. The concentration of IgGs did not differ between the sexes. Although only marginally significant, cellular components of the immune system seem to be more affected by sex than humoral components. In contrast to theoretical predictions (Folstad and Karter 1992), we found male greater sac-winged bats to invest more resources in immunity than females by having more immune cells and maintain higher functions of the complement mediated innate immunity measured by BKA. One likely explanation for this pattern is that males of this species may be more prone to infections than females and thus have to allocate more resources into components of the immune system. However, females of various bat species have been found to be more parasitized than males (e.g. Zahn and Rupp 2004; Christe et al. 2007), probably because clustering behaviour of females facilitates horizontal transmission of pathogens. In contrast, males of many bat species roost solitary. Yet, in greater sac-winged bats neither males nor females form clusters in roosts, but keep a minimal individual distance of 5-8 cm between each other (Bradbury and Emmons 1974). Thus, the risk for horizontal transmissions of pathogen should be low in this species, irrespective of the sex. Furthermore, if one sex is generally more exposed to diseases than the other, this should also be reflected in a higher level of immunoglobulins; a parameter that mirrors the cumulative contact of individuals with pathogens. In our study, IgG concentrations did not differ between males and females and thus, sex-specific pathogen susceptibility is unlikely.

Besides sex, we hypothesised age as a factor that might influence immunity. As age may impair the physiological functions of an organism due to senescence (Monaghan et al. 2008), we predicted that older bats differ in immune parameters compared to young bats. Indeed, we found that measures of the immune system were related to age in greater sac-winged bats. The number of immune cells decreased with increasing age in both males and females. On the one hand, this may be caused by a decrease in thymus size with age, and thus a reduced production of naïve T-cells (Haynes et al. 2000). On the other hand, the disappearance of individuals with high WBC counts may as well be caused by a selective

process, where individuals with infections are eliminated from the population. Our observation that individuals with high WBC counts are less likely present in the colonies half a year after sampling compared to individuals with low WBC count is in agreement with this idea. As individuals that were not present during both of the two visits at the colonies most likely died, high WBC counts may account for a higher mortality than low WBC counts. Studies on humans showed that individuals with WBC counts above or below average have higher mortality risks, yet the underlying cause for this pattern is still under debate (Ruggiero et al. 2007). In *S. bilineata* it remains to be investigated whether a high WBC count in bats is directly linked to an increased mortality due to higher overall costs of maintaining high number of immune cells in the plasma, or whether WBC count mirrors the current infection state of an individual and is therefore indirectly associated with mortality caused by pathogens.

Since we were not able to conduct functional assays of immune cells, we tested the competence of the constitutive humoral immune components by measuring the bacterial killing ability (BKA) of the plasma against *Escherichia coli*. Similarly to WBC count, we found that BKA was linked with the likelihood for disappearance of an individual from the colony. Although not associated with age, bats older than one year and with high BKA were more likely to disappear from the colonies than individuals with low BKA. Humoral immune components are often regarded as relatively inexpensive compared to cellular aspects (Lee 2006). Our results however suggest that maintaining a high level of constitutive humoral immunity may either have a disadvantage and thus to a certain extent be selected against, or similarly to WBC may reflect a current infection. Besides the direct energetic costs that may lead to trade-offs with other life-history traits, maintaining a high level of immune functions may increase the risk of autoimmunity (Balomenos and Martinez 2000; Viau and Zouali 2005). This is particularly the case for the activity of immune cells. For example, high rates of division and antibody production by B cells can lead to autoimmune diseases such as Lupus erythematosus in humans (Grammer and Lipsky 2003). Autoimmunity is mainly promoted by the increased production of antinuclear antibodies that usually bind foreign proteins, but may also be produced against antigens of the organism itself. In their study on Soay sheep, Graham and colleagues (2010) argued that the concentrations of antinuclear antibodies are positively correlated with overall antibody production and thus higher overall levels of antibodies may be beneficial. Here, we measured immunoglobulin G as a proxy for the overall concentration of antibodies for an animal. We found a positive effect of age on IgG concentration in both

sexes. By finding older individuals to have higher IgG concentrations than young ones, we found supporting evidence for the hypothesis that high antibody concentration may be a consequence rather than a cause of longevity (Graham et al. 2010). However, in contrast to antinuclear antibodies in Soay sheep (Graham et al. 2010), IgG concentration in *S. bilineata* did not predict survival. Individuals with higher levels of IgGs were not more or less likely to be encountered in colonies half a year after sampling compared to bats with low levels.

In conclusion, we provide first evidence for a correlation between age and measures of the immune system in a population of free-ranging bats. Our findings suggest that measures of the immune system vary between male and female bats, and also with age. Furthermore, we found both aspects of the cellular and humoral immune system to predict survival of bats. Individuals with relatively high age-specific levels of WBC and BKA were more likely to disappear from colonies than individuals with low levels. Therefore, high levels of immunity may be selected against, possibly due to high energetic costs or the increased risks of autoimmunity. To the best of our knowledge, this is the first study on how several aspects of the immune system are connected with survival in a free-ranging mammal.

Acknowledgements

We are grateful to Ivailo M. Borissov and Daniel Lewanzik for help in the field, and to Katja Pohle for her help during laboratory analyses. We thank the Organization for Tropical Studies (OTS) for allowance to use their facilities and for providing logistic support and the Costa Rican authorities for granting permission to conduct research. Financial support was provided by the Leibniz Institute for Zoo and Wildlife Research.

References

Allen, L. C., Turmelle, A. S., Mendoca, 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*). *Journal of Comparative Physiology B* **179**, 315-323.

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

Balomenos, D. and Martinez, A. (2000) Cell-cycle regulation in immunity, tolerance and autoimmunity. *Immunology Today* **21**, 551-555.

Belloni, V., Faivre, B., Guerreiro, R., Arnoux, E., Bellenger, J. and Sorci, G. (2010) Suppressing an anti-inflammatory cytokine reveals a strong age-dependent survival cost in mice. *Plos One* **5**, e12940.

Blount, D. G., Pritchard, D. I. and Heaton, P. R. (2005) Age-related alterations to immune parameters in Labrador retriever dogs. *Veterinary Immunology and Immunopathology* **108**, 399-407.

Bradbury, J. W. and Emmons, L. H. (1974) Social organization of some Trinidad bats I. Emballonuridae. *Zeitschrift für Tierpsychologie* **36**, 137-183.

Brunet-Rossinni, A. K. and Austad, S. N. (2004) Ageing studies on bats: a review. *Biogerontology* **5**, 211-222.

Christe, P., Arlettaz, R. and Vogel, P. (2000) Variation in intensity of a parasitic mite (*Spinturnix myoti*) in relation to the reproductive cycle and immunocompetence of its bat host (*Myotis myotis*). *Ecology Letters* **3**, 207-212.

Christe, P., Glaizot, O., Evanno, G., Bruyndonckx, N., Devevey, G., Yannic, G., Patthey, P., Maeder, A., Vogel, P. and Arlettaz, R. E. L. (2007) Host sex and ectoparasites choice: preference for, and higher survival on female hosts. *Journal of Animal Ecology* **76**, 703-710.

Drexler, J. F., Corman, V. M., Muller, M. A., Maganga, G. D., Vallo, P., Binger, T., Gloza-Rausch, F., Rasche, A., Yordanov, S., Seebens, A., Oppong, S., Sarkodie, Y. A., Pongombo, C., Lukashev, A. N., Schmidt-Chanasit, J., Stocker, A., Carneiro, A. J. B., Erbar, S., Maisner, A., Fronhoffs, F., Buettner, R., Kalko, E. K. V., Kruppa, T., Franke, C. R., Kallies, R., Yandoko, E. R. N., Herrler, G., Reusken, C., Hassanin, A., Kruger, D. H., Matthee, S., Ulrich, R. G., Leroy, E. M. and Drosten, C. (2012) Bats host major mammalian paramyxoviruses. *Nature Communications* **3**.

Eraud, C., Jacquet, A. and Faivre, B. (2009) Survival cost of an early immune soliciting in nature. *Evolution* **63**, 1036-1043.

Folstad, I. and Karter, A. J. (1992) Parasites, bright males, and the immunocompetence handicap. *American Naturalist* **139**, 603-622.

Frasca, D., Riley, R. L. and Blomberg, B. B. (2005) Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans. *Seminars in Immunology* **17**, 378-384.

Ginaldi, L., Loreto, M. F., Corsi, M. P., Modesti, M. and De Martinis, M. (2001) Immunosenescence and infectious diseases. *Microbes and Infection* **3**, 851-857.

Graham, A. L., Hayward, A. D., Watt, K. A., Pilkington, J. G., Pemberton, J. M. and Nussey, D. H. (2010) Fitness correlates of heritable variation in antibody responsiveness in a wild mammal. *Science* **330**, 662-665.

Grammer, A. C. and Lipsky, P. E. (2003) B cell abnormalities in systemic Lupus erythematosus. *Arthritis Research Therapy* **5**, S22-S27.

Hanssen, S. A., Hasselquist, D., Folstad, I. and Erikstad, K. E. (2004) Costs of immunity: immune responsiveness reduces survival in a vertebrate. *Proceedings of the Royal Society B* **271**, 925-930.

Haynes, B. F., Markert, M. L., Sempowski, G. D., Patel, D. D. and Hale, L. P. (2000) The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. *Annual Review of Immunology* **18**, 529-560.

Kondratov, R. V., Kondratova, A. A., Gorbacheva, V. Y., Vykhovanets, O. V. and Antoch, M. P. (2006) Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes & Development* **20**, 1868.

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. *Emerging Health Threats Journal* **4**, 7159.

Lee, K. A. (2006) Linking immune defenses and life history at the levels of the individual and the species. *Integrative and Comparative Biology* **46**, 1000-1015.

Lehmann, T. (1993) Ectoparasites: direct impact on host fitness. *Parasitology Today* **9**, 8-13.

Linton, P. J., Lustgarten, J. and Thoman, M. (2006) T cell function in the aged: Lessons learned from animal models. *Clinical and Applied Immunology Reviews* **6**, 73-97.

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. and Reeder, D. A. M. (2011)

Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. *Nature* **480**, 376-378.

Monaghan, P., Charmantier, A., Nussey, D. H. and Ricklefs, R. E. (2008) The evolutionary ecology of senescence. *Functional Ecology* **22**, 371-378.

Monath, T. P. (1999) Ecology of Marburg and Ebola viruses: Speculations and directions for future research. *Journal of Infectious Diseases* **179**, S127-S138.

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

Moore, M. S., Reichard, J. D., Murtha, T. D., Zahedi, B., Fallier, R. M. and Kunz, T. H. (2011) Specific alterations in complement protein activity of little brown myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. *Plos One* **6**, e27430.

Nagy, M., Heckel, G., Voigt, C. C. and Mayer, F. (2007) Female-biased dispersal and patrilocal kin groups in a mammal with resource-defence polygyny. *Proceedings of the Royal Society B* **274**, 3019-3025.

Noreen, E., Bourgeon, S. and Bech, C. (2011) Growing old with the immune system: a study of immunosenescence in the zebra finch (*Taeniopygia guttata*). *Journal of Comparative Physiology B* **181**, 649-656.

Nunn, C. L. (2002) A comparative study of leukocyte counts and disease risk in primates. *Evolution* **56**, 177-190.

Nunn, C. L., Gittleman, J. L. and Antonovics, J. (2000) Promiscuity and the primate immune system. *Science* **290**, 1168-1170.

Nunn, C. L., Gittleman, J. L. and Antonovics, J. (2003) A comparative study of white blood cell counts and disease risk in carnivores. *Proceedings of the Royal Society B* **270**, 347-356.

Nussey, D. H., Watt, K., Pilkington, J. G., Zamoyska, R. and McNeilly, T. N. (2012) Age-related variation in immunity in a wild mammal population. *Aging Cell* **11**, 178-180.

Pearce, R. D., O'Shea, T. J. and Wunder, B. A. (2008) Evaluation of morphological indices and total body electrical conductivity to assess body composition in big brown bats. *Acta Chiropterologica* **10**, 153-159.

R Development Core Team. (2010) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org

Reynolds, D. S., Sullivan, J. C. and Kunz, T. H. (2009) Evaluation of total body electrical conductivity to estimate body composition of a small mammal. *The Journal of Wildlife Management* **73**, 1197-1206.

Ricklefs, R. E. and Wikelski, M. (2002) The physiology/life-history nexus. *Trends in Ecology & Evolution* **17**, 462-468.

Ross, P. S., Pohajdak, B., Bowen, W. D. and Addison, R. F. (1993) Immune function in free-ranging harbor seal (*Phoca vitulina*) mothers and their pups during lactation. *Journal of Wildlife Diseases* **29**, 21-29.

Ruggiero, C., Metter, E. J., Cherubini, A., Maggio, M., Sen, R., Najjar, S. S., Windham, G. B., Ble, A., Senin, U. and Ferrucci, L. (2007) White blood cell count and mortality in the Baltimore Longitudinal Study of Aging. *Journal of the American College of Cardiology* **49**, 1841-1850.

Saino, N., Bolzern, A. M. and Møller, A. P. (1997) Immunocompetence, ornamentation, and viability of male barn swallows (*Hirundo rustica*). *Proceedings of the National Academy of Sciences* **94**, 549.

Schinnerl, M., Aydinonat, D., Schwarzenberger, F. and Voigt, C. C. (2011) Hematological survey of common neotropical bat species from Costa Rica. *Journal of Zoo and Wildlife Medicine* **42**, 382-391.

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.

Sheldon, B. C. and Verhulst, S. (1996) Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution* **11**, 317-321.

Stöbel, K., Schonberg, A. and Staak, C. (2002) A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi sl in zoo animals. *International Journal of Medical Microbiology* **291**, 88-99.

Tieleman, B. I., Williams, J. B., Ricklefs, R. E. and Klasing, K. C. (2005) Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. *Proceedings of the Royal Society B* **272**, 1715-1720.

Utsuyama, M., Hirokawa, K., Kurashima, C., Fukayama, M., Inamatsu, T., Suzuki, K., Hashimoto, W. and Sato, K. (1992) Differential age-change in the numbers of CD4+ CD45RA+ DC4+ CD29+ T cell subsets in human peripheral blood. *Mechanisms of Ageing and Development* **63**, 57-68.

Viau, M. and Zouali, M. (2005) B-lymphocytes, innate immunity, and autoimmunity. *Clinical Immunology* **114**, 17-26.

Voigt, C. C. and Schwarzenberger, F. (2008) Reproductive endocrinology of a small tropical bat (female *Saccopteryx bilineata*; Emballonuridae) monitored by fecal hormone metabolites. *Journal of Mammalogy* **89**, 50-57.

Voigt, C. C., Streich, W. and Dehnhard, M. (2007) Assessment of fecal testosterone metabolite analysis in free-ranging *Saccopteryx bilineata* (Chiroptera : Emballonuridae). *Acta Chiropterologica* **9**, 463-475.

Wellehan, J. F. X., Green, L. G., Duke, D. G., Bootorabi, S., Heard, D. J., Klein, P. A. and Jacobson, E. R. (2009) Detection of specific antibody responses to vaccination in variable flying foxes (*Pteropus hypomelanus*). *Comparative Immunology, Microbiology and Infectious Diseases* **32**, 379-394.

Wibbelt, G., Moore, M. S., Schountz, T. and Voigt, C. C. (2010) Emerging diseases in Chiroptera: why bats? *Biology Letters* **6**, 438-440.

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., Peel, A. J., Kamins, A. O., Fahr, J., Ntiamoa-Baidu, Y., Suu-Ire, R., Breiman, R. F., Epstein, J. H., Field, H. E. and Cunningham, A. A. (2012) A framework for the study of zoonotic disease emergence and its drivers: spillover of bat pathogens as a case study. *Philosophical Transactions of the Royal Society B* **367**, 2881-2892.

Zahn, A. and Rupp, D. (2004) Ectoparasite load in European vespertilionid bats. *Journal of Zoology* **262**, 383-391.

Zuk, M. and Stoehr, A. M. (2002) Immune defense and host life history. *American Naturalist* **160**, S9-S22.

Chapter III

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

(Published in Journal of Experimental Biology)

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

Karin Schneeberger, Gábor Á. Czirják, Christian C. Voigt

Abstract

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 infections as part of the activation of an immune response, leading to oxidative damage on 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 caused by low oxidative damage of organs. Here, we ask if 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 bacterial derived lipopolysaccharides (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 24h, whereas there was no significant change in leukocyte counts in control animals. The biological antioxidant potential (BAP) remained the same in both groups, but reactive oxygen metabolites (ROM) 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: Oxidative stress, immune response, bats, mammals

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 thus causes senescence in cells and thus is hypothesised to be an important modulator of life-history trade-offs in vertebrates (Costantini 2008; Nussey et al. 2009) and has therefore 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 both experimental and correlative studies do not always support the hypothesis that high oxidative stress leads to lower life-spans (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 low mass-specific metabolic rates (Pearl 1928; Harman 1955; Sacher 1959). Evidence for this free-radical theory of ageing suggested by 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 rate (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 due to their ability of powered flight (Munshi-South and Wilkinson 2010). Thus, the question arises if the high mass-specific metabolic rate of bats produces more pro-oxidants than those 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? First 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 machineries for damages caused by oxidation. It has recently been shown that genes regulating repair mechanisms of DNA damage is 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 less 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- 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) or 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*; Geoffroy) 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; Moore et al. 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 a higher mitochondrial activity and consequently to an increased ROS production (Finkel and Holbrook 2000). Additionally, different white blood cell subtypes involved in immune responses produce ROS to directly kill pathogens (Droege 2002), and to enhance the activation of T-lymphocytes (Droege 2002; Reth 2002). Thus, ROS have a signalling function during 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 should 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- and antioxidants are inconsistent (Costantini and Møller 2009). Furthermore, some of these studies only involve either pro- 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 both 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 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 5 out of 6 species. Furthermore, in contrast to many birds (Chaudhuri and Chatterjee 1969), bats - just like haplorhine primates, including humans (*Homo sapiens*; Linnaeus), capybaras (*Hydrochoerus hydrochaeris*, Linnaeus) and guinea pigs (*Cavia porcellus*; Linnaeus) - 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 lipopolisaccharides (LPS) and the resulting immune response leads to a change in reactive oxygen metabolites (ROM) representing total ROS produced and antioxidant level, expecting the concentration of ROM to increase and the level of antioxidants to decrease in order to avoid oxidative stress in bats.

Materials and Methods

We captured 12 *Carollia perspicillata* (6 males and 6 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 one week after the bats have been caught in 2011 and 2012 respectively. We marked bats individually and kept them in an outdoor flight cage (3.4 x 6.1 x 2.5 m) where they were fed *ad libitum* with banana and papaya and provided with water. The experiment started after all bats were habituated 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, Pesola, Switzerland). We took an initial blood sample of approximately 60 µl from each individual by punctuating the antibrachial vein with a sterile needle (#C721.1, Carl Roth GmbH, Germany) and by transferring blood drops into heparinised capillary tubes (#521-9100, VWR, 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 1mg/ml LPS (*Escherichia coli*, # L2630, Sigma Aldrich, Germany) in phosphate buffered saline solution (PBS; #L1825, Biochrom AG, Germany) using a sterile disposable syringe (# 0053.1, Carl Roth GmbH, Germany). 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 makers (Victor et al. 2004), leading to oxidative damage (Skibska et al. 2005). Individuals of the control group were injected with 50 µl PBS without the antigen. After 24 h post-injection, we

weighed each bat again and took an additional blood sample. Due to limitation in sample volume, we were restricted to measure 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 to detect 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 collecting the final blood sample.

Blood smears were stained with May-Gruenwald's solution (#T863.2, Carl Roth GmbH, Germany) and Giemsa (#T862.1, Carl Roth GmbH, Germany). We manually estimated total WBC count by counting the cells in 10 visual fields with a microscope under 200x magnifications (Schneeberger et al. 2013).

We measured markers of oxidative stress (dROM and BAP) using the Free Radical Analytical System (FRAS4 evolvo; H&D srl, Italy) following the instructions provided by the distributor. We measured the concentration of ROMs using dROM-kits, which represents the total hydroperoxide level in plasma that are created during peroxidation of amino acids, lipids and proteins, representing free radicals from which they are formed. We added 10 µl of plasma to a buffered chromogen, where the derivates of ROMs form a coloured compound which 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 direct proportional to the concentration of ROMs and is expressed as U Carr. 1 U Carr is equivalent to 0.08 mg/dl 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.

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 if the delay between capturing and handling had an effect on measurements of cellular immune response and oxidative stress. We did not find such influence on WBC count (Spearman rank correlation; rho=-0.160; p=0.157), dROM (rho=0.046; p=0.684) or BAP (rho=0.067; p=0.559), excluding the potential of capturing and handling stress to confound our subsequent analysis.

To test whether potential changes in measures of oxidative stress and WBC count is accounted to treatment with LPS or PBS respectively, 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 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 if 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 (X^2=12.56; p=0.006), but not with sex (X^2=0.22; p=0.639). Body mass decreased 24h 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 (X^2=11.34; p=0.010), but not between males and females (X^2=1.11; p=0.737). WBC counts increased significantly 24h 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 ± one standard deviation) and BAP was 2'294 ± 0.48 µMol/l before treatment. dROM was significantly linked to treatment and day of sampling (X^2=12.52; p=0.006), but not to sex (X^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 (X^2=3.38; p=0.336) nor to sex (X^2<0.001; p>0.999).

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

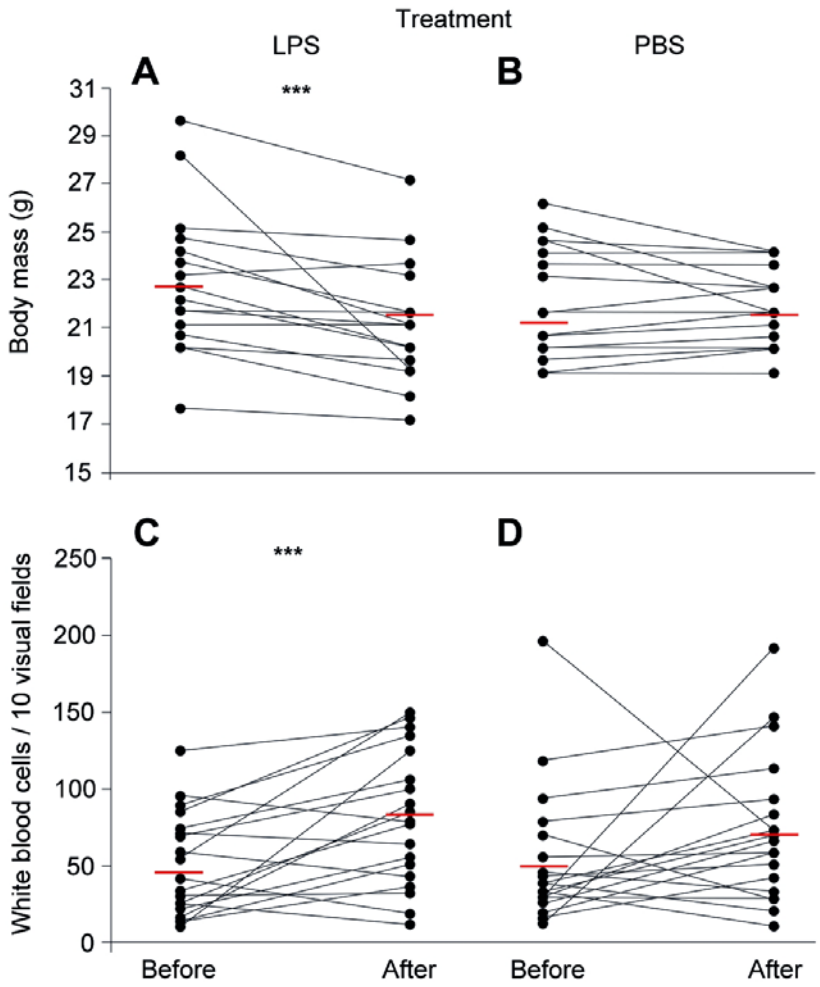


Fig. 1: Body mass decreases in individuals of the experimental group 24h post-treatment with lipopolysaccarides (LPS; A), but not of the control group injected with phosphate buffered saline solution (PBS; B). White blood cell (WBC) counts per 10 visual fields increase in animals of the experimental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

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 (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 if mounting an immune response in bats leads to an increase in reactive oxygen metabolites (ROMs), representing the total ROS created, and if this changes the concentration of antioxidants to mitigate oxidative stress. To our best knowledge this is the first study on the effect of mounting an immune response on oxidative stress markers in a free-ranging mammal.

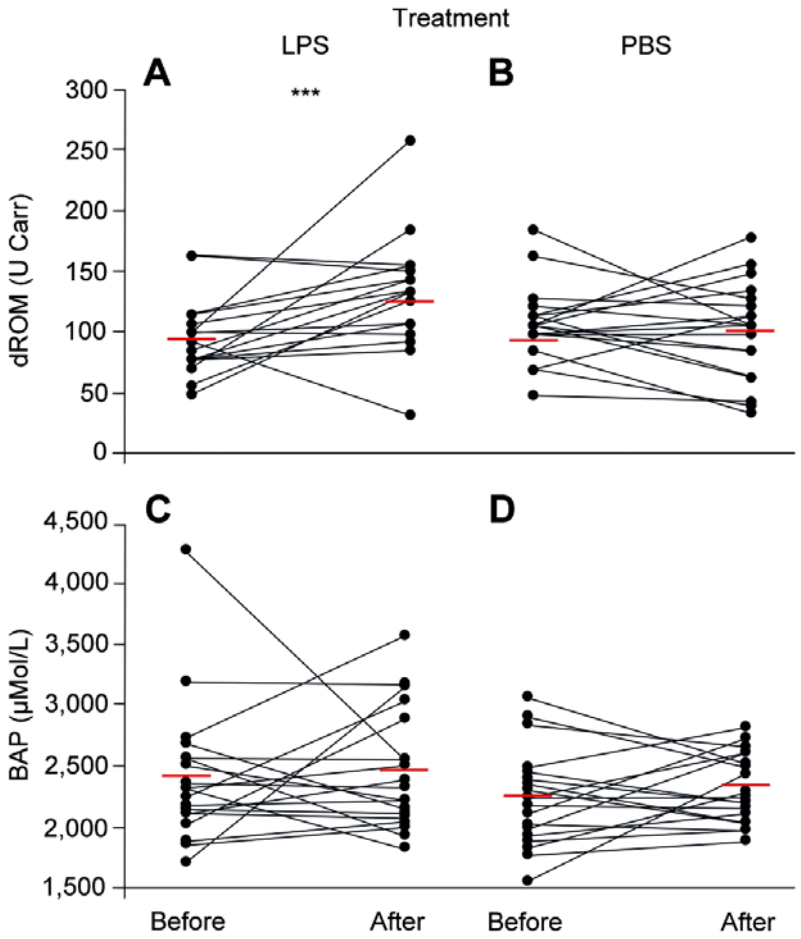


Fig. 2: Concentration of reactive oxygen metabolites (dROM) increases in bats 24h after injection with lipopolysaccharide (LPS; A), but not with phosphate buffered solution (PBS) as a control (B). Antioxidant concentration (BAP) remained the same in both the experimental (C) and control group (D). Red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

Total WBC count increased significantly in the experimental group 24 h after the 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 in body mass and associated decreased food ingestion might be the result of LPS-caused sickness behaviour. In mice, it has been shown previously 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 with an increased

concentration of ROMs found 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 (Droege 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 rather a consequence of physiological processes involved in sickness behaviour, such as elevated metabolic rates (Finkel and Holbrook 2000) as well as ROS produced to enhance the cellular immune response and to directly kill pathogens (Droege 2002; Reth 2002). This is supported by the finding that dROM correlated positively with WBC counts. 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;* Linnaeus; Costantini and Dell'Omo 2006). In red-legged partridges (*Alectoris rufa*; Linnaeus), the total antioxidant concentration remained the same before and after injection with phytohaemagglutinin (Perez-Rodriguez et al. 2008), but increased in greenfinches (*Carduelis chloris*; Linnaeus; Hõrak et al. 2007). The same was true for chicken (*Gallus gallus domesticus*; Linnaeus) injected with LPS (Cohen et al. 2007): There was no change in the level of various antioxidants before and 24h 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 24h (Percival and Sims 2000). Similarly to these studies, we found that the antioxidant concentration did not change after treatment with LPS in *C. perspicillata*. Potentially, an antioxidant barrier needs more time than 24 h to be raised, as there may be a time lag between increase of free-radicals and the according antioxidant response (Hõrak and Cohen 2010; Meitern et al. 2013). In their study on red-legged

partridges, Perez-Rodriguez and his colleagues (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, only 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 other factors 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 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 (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, *C. perspicillata* shows with a mean baseline BAP of 2.294 µMol/l a rather moderate concentration of antioxidants comparable to the level observed in rats (1,874 µMol/l; Iwata et al. 2010) and mice (2,896 µMol/l; Maruoka and Fujii 2012).

We conclude that an immune response can up-regulate some markers frequently associated with oxidative stress even in a long-lived mammal that is known to have a reduced pro-oxidant production compared to terrestrial mammals (Brunet-Rossinni 2004; Wilhelm Filho et al. 2007). Thus, bats may suffer from longterm consequences of elevated oxidative stress after episodes of acute or during chronic infections. The long life-span 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.

Acknowledgement

We are grateful to Daniel Lewanzik, Oliver Lindecke and Tobias Teige for help in the field. We thank the Organization for Tropical Studies (OTS) for allowance to use their facilities and for providing logistic support and the Costa Rican authorities for granting permission to conduct this research [186-2012-SINAC]. This study has been funded by the Deutsche Forschungsgemeinschaft (DFG Vo 890/25).

References

Adelman, R., Saul, R. L. and Ames, B. N. (1988) Oxidative damage to DNA: relation to species metabolic rate and life span. *Proceedings of the National Academy of Sciences* **85**, 2706-2708.

Allen, L. C., Turmelle, A. S., Mendoca, 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*). *Journal of Comparative Physiology B* **179**, 315-323.

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

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. *The FASEB Journal* **14**, 312-318.

Birney, E. C., Jenness, R. and Ayaz, K. M. (1976) Inability of bats to synthesize 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. *Mechanisms of Ageing and Development* **125**, 11-20.

Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. *Physiological Reviews* **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. *Comparative Biochemistry and Physiology B* **147**, 110-121.

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

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

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

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

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

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

Drexler, J. F., Corman, V. M., Muller, M. A., Maganga, G. D., Vallo, P., Binger, T., Gloza-Rausch, F., Rasche, A., Yordanov, S., Seebens, A., Oppong, S., Sarkodie, Y. A., Pongombo, C., Lukashev, A. N., Schmidt-Chanasit, J., Stocker, A., Carneiro, A. J. B., Erbar, S., Maisner, A., Fronhoffs, F., Buettner, R., Kalko, E. K. V., Kruppa, T., Franke, C. R., Kallies, R., Yandoko, E. R. N., Herrler, G., Reusken, C., Hassanin, A., Kruger, D. H., Matthee, S., Ulrich, R. G., Leroy, E. M. and Drosten, C. (2012) Bats host major mammalian paramyxoviruses. *Nature Communications* **3**.

Droege, W. (2002) Free radicals in the physiological control of cell function. *Physiological Reviews* **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. *Mechanisms of Ageing and Development* **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. *General and comparative endocrinology* **168**, 466-473.

Halliwell, B., Bomford, A. B., Stern, A., Golenser, J., Chevion, M., Callahan, H. L., Aldunate, J., Morello, A., Cross, C. E., Balasubramanian, K. A., Mathan, V. I., and Eze, V. O. (1993). *Free radicals in tropical diseases*. Routledge.

Harman, D. (1955). *Aging: a theory based on free radical and radiation chemistry*. University of California Radiation Laboratory.

Hõrak, P. and Cohen, A. (2010) How to measure oxidative stress in an ecological context: methodological and statistical issues. *Functional Ecology* **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. *The American Naturalist* **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. *Physiological Reviews* **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. *Journal of Health Science* **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. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **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. *Emerging Health Threats Journal* **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. *Journal of leukocyte biology* **57**, 440-449.

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J. H., Wang, H., Crameri, G., Hu, Z. and Zhang, H. (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. and Reeder, D. A. M. (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. *Journal of Physical Therapy Science* **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. *The Journal of experimental biology* **216**, 2713-2721.

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

Moore, M. S., Reichard, J. D., Murtha, T. D., Zahedi, B., Fallier, R. M. and Kunz, T. H. (2011) Specific alterations in complement protein activity of little brown myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. *Plos One* **6**, e27430.

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. *Comparative Biochemistry and Physiology B* **147**, 492-497.

Munshi-South, J. and Wilkinson, G. S. (2010) Bats and birds: Exceptional longevity despite high metabolic rates. *Ageing Research Reviews* **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. *Functional Ecology* **23**, 809-817.

Pearl, R. (1928). *The rate of living*. University of London Press.

Percival, S. S. and Sims, C. A. (2000) Wine modifies the effects of alcohol on immune cells of mice. *Journal of Nutrition* **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*). *Journal of Experimental Biology* **211**, 2155-2161.

R Development Core Team (2010) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.com

Reth, M. (2002) Hydrogen peroxide as second messenger in lymphocyte activation. *Nature Immunology* **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? *International Journal of Biochemistry & Cell Biology* **34**, 1449-1460.

Sacher, G. 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 in Ecology & Evolution* **11**, 317-321.

Skibska, B., Józefowicz-Okonkwo, G. and Goraca, A. (2005) Protective effects of early administration of alpha-lipoic acid against lipopolysaccharide-induced plasma lipid peroxidation. *Pharmacological Reports* **58**, 399-404.

Sohal, R. S., Svensson, I. and Brunk, U. T. (1990) Hydrogen peroxide production by liver mitochondria in different species. *Mechanisms of Ageing and Development* **53**, 209-215.

Sohal, R. S. and Weindruch, R. (1996) Oxidative stress, caloric restriction, and aging. *Science* **273**, 59-63.

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. *Proceedings of the National Academy of Sciences* **77**, 2777-2781.

Victor, V. M., Rocha, M. and De la Fuente, M. (2004) Immune cells: free radicals and antioxidants in sepsis. *International Immunopharmacology* **4**, 327-347.

Wibbelt, G., Moore, M. S., Schountz, T. and Voigt, C. C. (2010) Emerging diseases in Chiroptera: why bats? *Biology Letters* **6**, 438-440.

Wilhelm Filho, D., Althoff, S. L., Dafre, A. L. and Boveris, A. (2007) Antioxidant defenses, longevity and ecophysiology of South American bats. *Comparative Biochemistry and Physiology C* **146**, 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., Peel, A. J., Kamins, A. O., Fahr, J., Ntiamoa-Baidu, Y., Suu-Ire, R., Breiman, R. F., Epstein, J. H., Field, H. E. and Cunningham, A. A. (2012) A framework for the study of zoonotic disease emergence and its drivers: spillover of bat pathogens as a case study. *Philosophical Transactions of the Royal Society B* **367**, 2881-2892.

Chapter IV

Frugivory reduces measures of oxidative stress in free-ranging bats

(Submitted)

Frugivory reduces measures of oxidative stress in free-ranging bats

Karin Schneeberger, Gábor Á. Czirjak, Christian C. Voigt

Abstract

Oxidative stress – an imbalance between damaging pro- and neutralising anti-oxidants – is regarded as the main cause of ageing in animals. Theory predicts that feeding on diets with high antioxidant content such as fruits should reduce oxidative stress. Bats are long-lived animals considering their size and cover a large variety of ecological niches. Therefore, we asked if measures of oxidative stress differ among bats with varying feeding habits. We measured reactive oxygen metabolites (ROM) representing total pro-oxidants produced and antioxidants in the plasma of 13 Neotropical bat species (127 individuals). Species with a fruit diet showed the lowest level of ROM, followed by omnivorous and animalivorous species. Plasma antioxidant levels did not differ among feeding habits. Potentially, frugivorous species ingest more antioxidants with food and thus are able to neutralise more pro-oxidants than in species not feeding on fruits. Possibly, the evolution of frugivory in bats may have been facilitated by the beneficial effects of dietary antioxidants on life-span and thus overall fitness. We showed for the first time that measures of oxidative stress vary according to dietary niche in free-ranging mammals, suggesting that the ecological niche of an animal may influence its rate of ageing. Furthermore, bats had significantly lower levels of ROM and higher antioxidant concentrations compared with shorter lived rodents.

Keywords Oxidative stress, dietary niche, bats, mammals, antioxidants, reactive oxygen species

Introduction

Reactive oxygen species (ROS) are produced as by-products during energy production in all aerobic organisms. It is estimated that more than 2% of oxygen consumed by cells is diverted to generate hydrogen peroxide and superoxide, among the most important ROS (Chance et al. 1979). These chemicals are highly reactive and can lead to oxidative damage by peroxidation of membrane fatty acid chains, loss of sulfhydryls and carbonylation in proteins and modification of DNA (Sohal et al. 1990; Sohal and Brunk 1992; Sohal and Weindruch 1996; Goyns 2002). These negative effects can be mitigated via raising an antioxidant barrier, which can either be diet-derived (exogenous) or enzymatic (endogenous). Antioxidants convert ROS into less reactive molecules and thus prevent ROS from damaging cell structures. An imbalance between antioxidants and ROS results in oxidative stress, which may impair the metabolism of an organism, causing cellular senescence (Rose et al. 2002). Oxidative stress has therefore been regarded as the main cause of ageing for cells and whole organisms for the last decades (Harman 1955; Liochev 2013). However, the free-radical theory of ageing has recently been under debate, as both experimental and correlative studies do not always support the hypothesis that high oxidative stress leads to lower life-spans (Speakman and Selman 2011; Selman et al. 2012).

Endogenously derived enzymatic antioxidants (e.g. superoxide dismutases and peroxidases) are highly efficient and form a first-line defence against pro-oxidants, mainly directly within the mitochondria, where ROS are produced (Balaban et al. 2005). Non-enzymatic chain breaking antioxidants produced *in vivo* such as ubiquinones, glutathione, uric acid and in most species vitamin C are the next level of defence against oxidative stress. However, the concentration of endogenous antioxidants is not sufficient to decrease the oxidative stress of an animal to a degree that is physiologically tolerable. Thus, diet-derived antioxidants like vitamin A and E, carotenoids, enzyme cofactors (Q10), nitrogen compounds and peptides play a major role in mitigating the negative effects of ROS. Although a large body of literature has been generated on the beneficial effects of dietary antioxidants, mainly in human literature (reviewed in Carocho and Ferreira 2013) and on carotenoids in birds (reviewed in Garratt and Brooks 2012), studies on other taxa have demonstrated opposite or neutral trends, and thus the role of dietary antioxidants remains rather unclear, particularly for free-ranging mammals.

The creation of ROS generally increases linearly with the amount of energy produced, i.e. with mass-specific metabolic rate, which is why animals with a high mass-specific

metabolic rate should show higher levels of oxidative stress and shorter life spans than species with low metabolic rates (Pearl 1928; Harman 1955; Sacher 1959). Bats seem to be an exception to this general rule. Although they have high mass-specific metabolic rates due to their ability of active flight (Munshi-South and Wilkinson 2010), they live on average 3.5 times longer than similar-sized terrestrial mammals (Austad and Fischer 1991; Wilkinson and South 2002). If oxidative stress is related to longevity in free-ranging mammals, one could ask if bats produce less ROS than similar sized terrestrial mammals, or if they have a particularly strong antioxidant barrier in order to mitigate negative effects of ROS, extending their life-span rather than leading to fast senescence as predicted by their metabolism.

Studies on oxidative stress in bats have been confined to single species and their results are rather inconsistent. So far, higher absolute concentrations of hydrogen peroxide have been found in brain, heart and lung tissue of little brown bats (*Myotis lucifugus*) compared to the concentration in tissues of similar sized white-footed mice (*Peromyscus leucopus*; Brunet-Rossinni 2004). However, absolute concentrations were lower than in the organs of the northern short-tailed shrew (*Blarina brevicauda*). When looking at the amount of ROS produced per unit of oxygen consumed, *M. lucifugus* had lower levels than the two terrestrial mammals of similar size. The author concluded that bats seem to have more efficient mitochondria than other mammals; an idea that has not been verified yet for any bat species. *Myotis lucifugus* did not differ in the activity of superoxide dismutase, an important antioxidant, from *B. brevicauda* and *P. leucopus* (Brunet-Rossinni 2004). However, five South American bat species had higher levels of both enzymatic and non-enzymatic antioxidants in their organs than similar-sized, terrestrial mammals (Wilhelm Filho et al. 2007). Thus, it remains unclear if bats in general differ in their oxidative stress from similar-sized, terrestrial mammals. Furthermore, it remains to be investigated what factors influence the levels of pro- and antioxidants in mammals.

We aimed at providing the first comprehensive study on the relationship between plasma oxidative stress and diet in free-ranging mammals by measuring pro- and antioxidant concentration of bat species with contrasting feeding habits. We hypothesised that species feeding on diets rich in fruits have lower levels of oxidative stress due to an increased consumption of dietary antioxidants. We furthermore aimed to compare the levels of pro- and antioxidants in bats with those documented for rats and mice that are of similar size than bats, but terrestrial and relatively short living.

Materials and methods

We caught bats within the vicinity of "La Selva" Biological Station (10°25'N; 84°00'W, Province Heredia, Costa Rica) in November and December 2012 between 5 pm and 10 pm at ground level using nylon mist nets (2.5 m height, Ecotone, Gdynia, Poland). Species were identified according to Timm and LaVal (1998). As it is not possible to distinguish between *Artibeus phaeotis* and *Artibeus watsoni* in the field, we will refer to these two species as *Artibeus c.f. watsoni* (Schinnerl et al. 2011). *Saccopteryx bilineata* were caught at dawn (5 am – 7 am) when individuals returned to their daytime roost or during the day directly in the roost using a hand net. Bats were placed individually in cotton bags and weighed using a spring balance (accuracy 0.5 g, Pesola; Switzerland). Sex and reproductive status was assessed and pregnant and lactating females were released immediately at the site of capture without any sample being taken. Juveniles were distinguished from adults by examining the degree of the epiphysial closure of the phalanges and also immediately released at the site of capture without further sampling. From all other bats, a small blood sample of no more than 5% of the total blood volume was taken by punctuating the antebrachial vein with a sterile needle (#C721.1, Carl Roth GmbH, Germany) and collecting the blood droplets with a heparinised capillary (#521-9100, VWR, Germany). The plasma was collected after centrifugation and stored at -80°C until further analysis. All bats were released at the site of capture.

In species with more than 5 captured individuals, we measured the concentration of reactive oxygen metabolites (ROM) in plasma, representing the overall ROS produced, using dROM-kits of the Free Radical Analytical System (FRAS4 evolvo; H&D srl, Italy), following the instructions provided by the distributor. This kit measures the total hydroperoxide level in plasma that is created during peroxidation of amino acids, lipids and proteins, representing free radicals from which they are formed. We added 10 µl of plasma to a buffered chromogen, where the derivates of ROMs form a coloured compound which 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. 1 U Carr is equivalent to 0.08 mg/dl hydrogen peroxide, which represent the total ROMs resulting of peroxidation chain reactions of amino acids, lipids and proteins. The biological antioxidant potential (BAP) 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 non-enzymatic 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 its antioxidant concentration expressed as mMol/l.

We used "R" version 2.13.1 for all statistical analysis (R Development Core Team 2010). Mean dROM and BAP was calculated for all species, and species were assigned to their dietary niche according to LaVal and Rodríguez (2002), depending on the relative proportion of fruits in the diet of the bats ("all fruits" - frugivorous, "partially fruits" – omnivorous, "no fruits" - animalivorous). Species of the genus *Carollia* were considered to be omnivorous, as recent studies have shown that although being specialised on *Piper* fruits, they include relatively large numbers of insects in their diet as well (Voigt et al. 2008; York and Billings 2009).

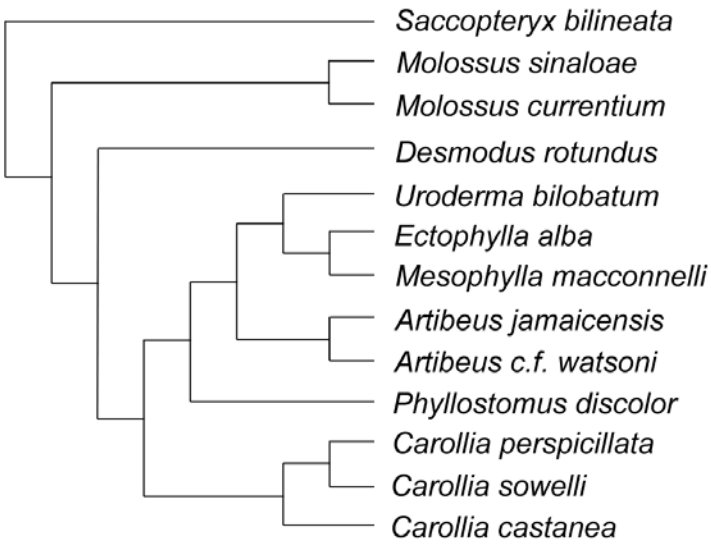


Fig. 1: Phylogenetic tree of species included in the study.

As two closely related species may share inherited characteristics from a common ancestor, species-specific data can not be considered as statistically independent (Price 1997). We therefore calculated phylogenetic generalised least squares models (PGLS; Pagel 1999) on the effect of the amount of fruits in the diet on oxidative stress parameters using the "gls" function of the package "nlme" (Pinheiro et al. 2009) and accounted for phylogeny using the "correlation" function of the package "ape" (Paradis et al. 2004). We used a phylogenetic tree modified after Jones and colleagues (Jones et al. 2002; Fig. 1). Details of the phylogeny of the genus *Carollia* was drawn from Hoffmann & Baker (Hoffmann and Baker 2003). We used the covariance matrix "corGrafen" (Grafen 1989), as in initial trials, this resulted in the lowest estimates of model AIC (Akaike's information

criterion). Accordingly, as branch lengths were unknown, we artificially computed them as suggested by Grafen (1989), with the length of a branch being the number of descending taxa minus 1. Although we only analysed species with 5 or more individuals, the exact number of individuals caught for each species still varied. We therefore weighted the data by sample size for each species divided by the total number of bats caught to account for heteroscedasticity of the variance. Residuals of the models were normally distributed, and for all analyses, we set the level of significance to α=0.05.

dROM and BAP of mice (*Mus musculus*), rat (*Rattus norvegicus*) and humans (*Homo sapiens*) were extracted from the literature (Iwata et al. 2010; Maruoka and Fujii 2012; Jansen and Ruskovska 2013). As the data were normally distributed, we used one-sample t-tests to assess the difference between bats and mice, rats and humans.

Results

We assessed the concentration of reactive oxygen metabolites (dROM) and the biological antioxidants potential (BAP) in plasma of 127 bats belonging to 13 species (Fig. 2). Five species fed exclusively on fruits (*Artibeus cf. watsoni*, *A. jamaicensis*, *Ectophylla alba*, *Mesophylla macconnelli*, and *Uroderma bilobatum*), four were omnivorous species feeding on both fruits and insects (*Carollia castanea*, *C. sowelli*, *C. perspicillata* and *Phyllostomus discolor*), and four were animalivorous species feeding either on insects (*Molossus currentium*, *M. sinaloae* and *Saccopteryx bilineata*) or on vertebrate blood (*Desmodus rotundus*).

BAP did not differ between feeding habits ($F_{2,10}$=0.88; p=0.44; Fig 3a), but the level of dROM varied significantly between species according to the proportion of fruits in their diet ($F_{2,10}$=11.0; p=0.003; Fig 3b). Frugivorous bats had a significantly lower level of dROM than omnivorous species that only partly incorporated fruits in their diet (t=-3.5; p=0.005) and animalivorous bats that did not feed on fruits at all (t=-3.9; p=0.003). Omnivorous species did not differ in dROM from animalivorous species (t=-0.4; p=0.68). Body mass did not correlate with dROM ($F_{1,11}$=2.24; p=0.163) or BAP ($F_{1,11}$=1.42; p=0.26).

dROM of bats was significantly lower than dROM of mice (t=-12.8; df=12; p<0.001), rats (t=-11.5; df=12; p<0.001) and humans (t=-55.1; df=12; p<0.001). BAP of bats was higher than BAP of rats (t=7.8; df=12; p<0.001) and humans (t=3.1; df=12; p=0.009), but did not differ significantly from BAP reported for mice (t=-0.52; df=12; p=0.61).

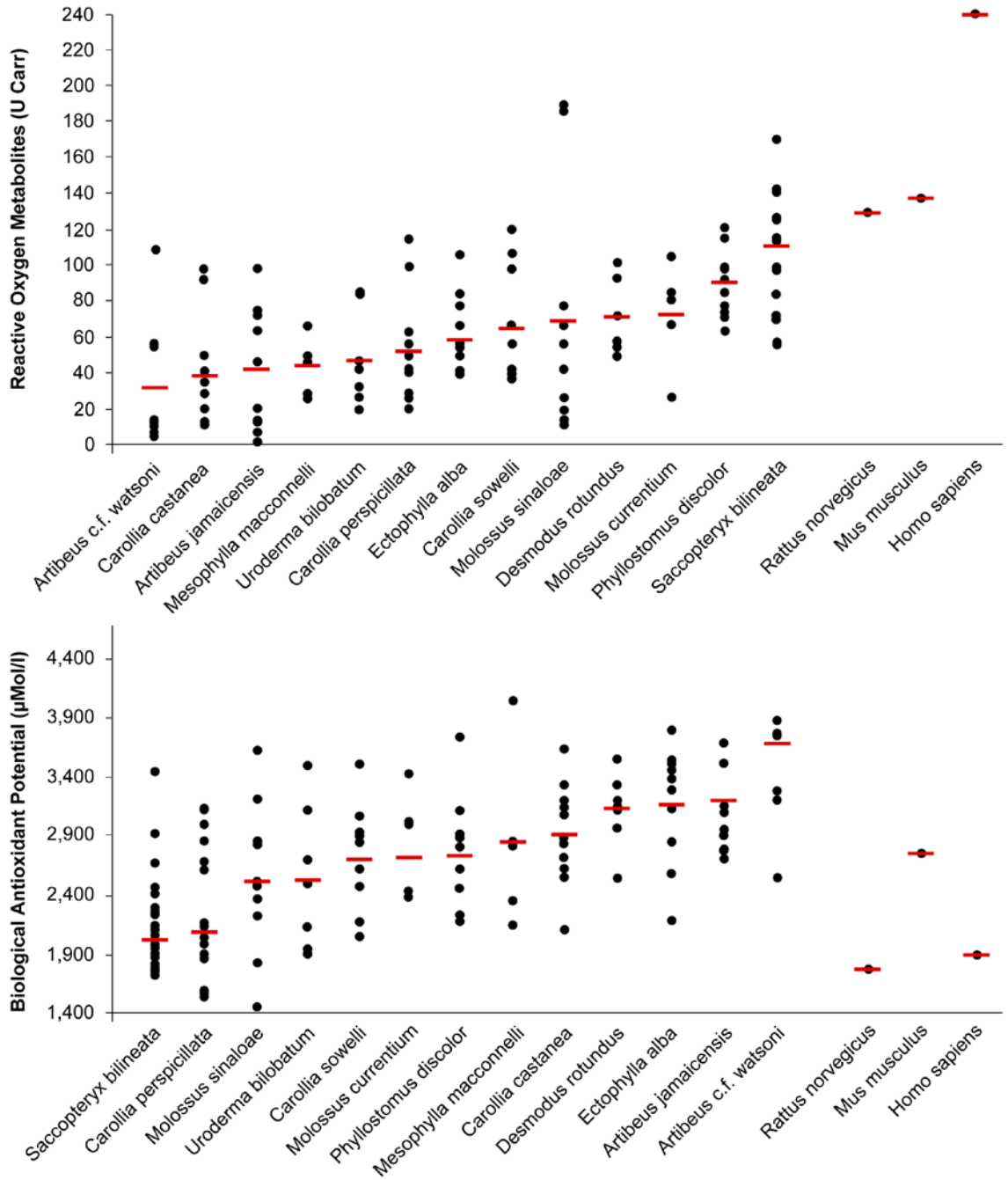


Fig. 2: Individual measures (black dots) and mean values (red bars) of concentrations of reactive oxygen metabolites (a) and biological antioxidant potential (b) sorted according to increasing average values for bats. For comparative purposes, corresponding values were also plotted for rodents and humans based on literature (Iwata et al. 2010; Maruoka and Fujii 2012; Jansen and Ruskovska 2013).

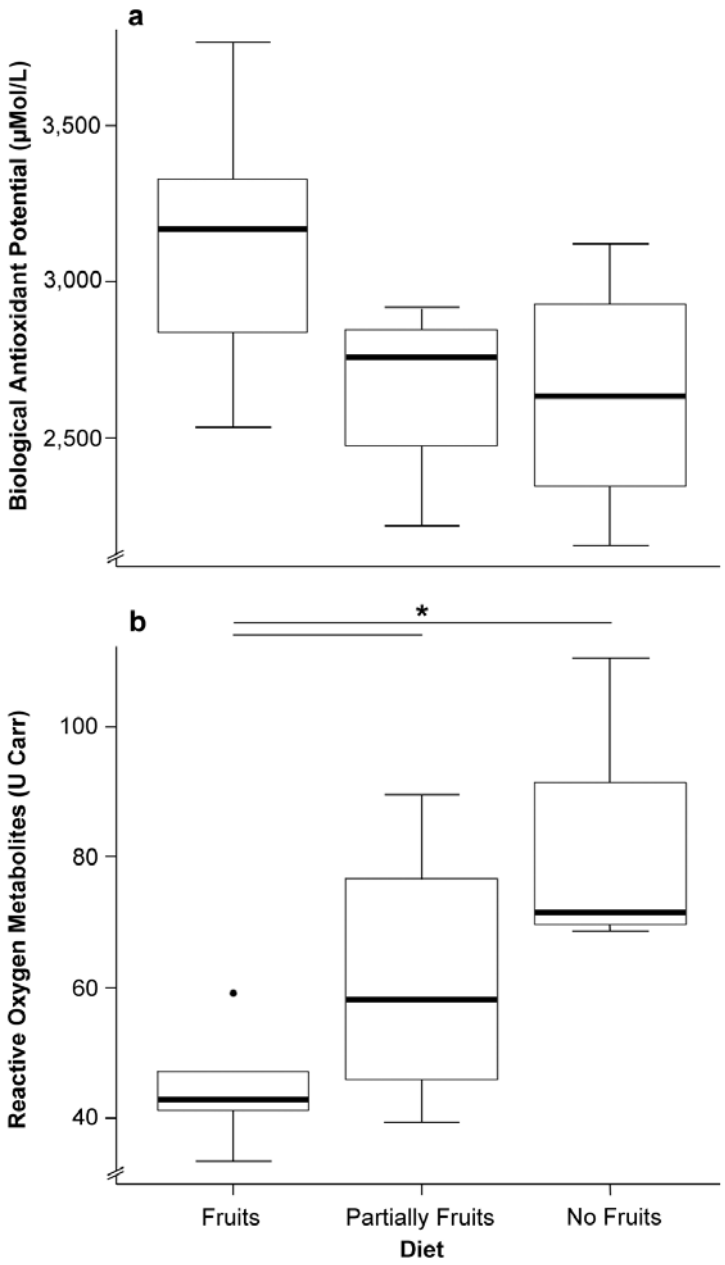


Fig. 3: Measures of oxidative stress for frugivorous (fruit diet), omnivorous (partially fruit diet) and animalivorous bat species (no fruit diet). Concentration of reactive oxygen metabolites differed significantly between dietary niches (a), whereas antioxidant concentration did not differ between dietary niches (b). Asterisks indicate significant differences at $p < 0.05$.

Discussion

The goal of our study was to determine if measures of oxidative stress differ among mammals with contrasting feeding habits, using bats as a model system. We assessed the mean concentration of reactive oxygen metabolites (ROM), reflecting overall reactive oxygen species (ROS) produced, and the biological antioxidant potential (BAP) in plasma

of 13 Neotropical bat species. We found that although BAP did not differ between dietary niches, ROM was lowest in species that fed exclusively on fruits, followed by species that included fruits partially in their omnivorous diet. Bats that did not include fruits in their diet had the highest level of ROM in their plasma. We argue that an antioxidant rich diet enabled frugivorous bats to reduce pro-oxidants more efficiently than insectivorous bats, i.e. higher levels of ROM in non-frugivorous bat species may result from a lowered defence of dietary antioxidants against pro-oxidants. Alternatively, insectivorous bats may as well produce higher levels of ROS which may lead in consequence to an overall higher concentration of ROM in plasma of insectivorous compared to frugivorous bats. Indeed, one of our previous studies showed that variations in immunological measures may be related to the ecological niche of bats (Schneeberger et al. 2013). Oxidative stress could be caused by species-specific immunological parameters, because white blood cells produce ROS to directly kill pathogens (Droege 2002) and to enhance the activation of T-lymphocytes (Droege 2002; Reth 2002). Results of our previous study on the immune system of Neotropical bats showed that insectivorous bats had the lowest white blood cell count, followed by frugivores and carnivores (Schneeberger et al. 2013). Surprisingly, we did not find insectivorous bats to have lower levels of ROM than other bats. Also, bats with an at least partial carnivorous diet (*Phyllostomus hastatus*, *Trachops cirrhosus* and *Desmodus rotundus*) and above-average high white blood cell counts have not exceedingly high concentrations of ROM compared to other bat species. Thus, variation in cellular immunity among dietary niches does not explain differences in plasma ROM levels between frugivorous and non-frugivorous species.

How do measures of oxidative stress in Chiroptera compare with other mammalian taxa and with longevity? Previous studies comparing measures of oxidative stress among bats and non-flying mammals have been rather inconsistent (Brunet-Rossinni 2004; Wilhelm Filho et al. 2007). Our study provides the first comprehensive dataset on this topic. We found that bats have lower levels of ROMs and higher concentrations of antioxidants than for example rats and mice, even though bats have a higher metabolic rate than rodents (Munshi-South and Wilkinson 2010). The high mass-specific metabolic rate of bats should in theory produce more ROS than the lower metabolic rate of rodents and thus should be reflected by higher concentrations of plasma ROMs. Contrasting to this prediction, bats had lower levels of ROM and thus seem to produce less ROS than mice, yet antioxidant levels were similar. This difference in measures of oxidative stress was apparent between bats and rodents even though rodents were kept under ideal conditions in

a laboratory setting, being fed a standardised diet with optimal levels of dietary antioxidants, which may explain partly why antioxidant levels did not differ between bats and rodents. Bats in general live 3.5 times as long as similar-sized terrestrial mammals (Austad and Fischer 1991; Wilkinson and South 2002). Data on longevity are known for five bat species of our dataset: *Artibeus jamaicensis* (10 years), *Carollia perspicillata* (12 years), *Desmodus rotundus* (19 years), *Phyllostomus discolor* (9 years) and *Saccopteryx bilineata* (6 years; Wilkinson and South 2002). Mice and rats live on average only 4 years under laboratory conditions (Turturro et al. 1999), and thus, although not statistically verified, this pattern is consistent with the idea that levels of oxidative stress are possibly linked to species longevity across larger taxonomic groups. However, bats and rodents do not only differ in their life span and mode of locomotion, but also with respect to their reproductive strategies (MacArthur 1967). Rats and mice, on the one hand, are r-strategists, producing high numbers of low-cost offspring. Bats, on the other hand, follow an extreme K-strategy. They produce only one or two offspring per year, and the newly born offspring have relatively high birth masses (about 30% of the mothers' body mass; Barclay et al. 2003). Species of our dataset are members of the family Phyllostomidae, Molossidae and Emballonuridae, which only have one pup per litter (Barclay et al. 2003). Two litters per year have been reported for *Uroderma bilobatum* (Baker and Clark 1987), *Ectophylla alba* (Timm 1982), *Artibeus jamaicensis* (Ortega and Castro-Arellano 2001), *A. c.f. watsoni* (Timm 1985), *Phyllostomus discolor* (Kwiecinski 2006) and species of the genus *Carollia* (Cloutier and Thomas 1992) which have a biannual, polyestrous cycle. Surprisingly, these species have rather low concentrations of ROM compared to species with only one litter per year. Thus, differences in the number of offspring may explain some of the differences across lager taxonomic groups, i.e. between Chiroptera and Rodentia, yet such differences fail to explain levels of oxidative stress among species within the order Chiroptera.

In the following paragraph we evaluate if dietary antioxidants could have played a role in the evolution of frugivory during the radiation of Chiroptera. Fossil records indicate that bats most likely evolved from nocturnal, arboreal, insect-eating mammals (Speakman 2001), and thus, insectivory was the initial feeding strategy of bats. Frugivory established twice during the evolution of bats (Dumont et al. 2003), namely in old-world fruit-bats (Pteropodidae) with 171 frugivorous species and members of the family Phyllostomidae endemic to the Neotropics with an estimated 96 bat species feeding on fruits. While old-world fruit-bats radiated from insectivorous species at a relatively early stage of the Chiropteran evolution, new-world fruit bats are a relatively newly derived clade (Simmons

and Geisler 1998). So far, it is unknown why bats partly shifted from insects to fruits. Possibly, nutritional properties of fruits were important drivers for the resource partitioning among co-existing bat species and the evolution of feeding habits (Dumont et al. 2003). Possibly, the shift in dietary preferences from insectivory to frugivory, i.e. towards antioxidant rich food, has been promoted by the beneficial effects of dietary antioxidants on longevity in ancestral bats. An increased lifetime fitness of pre-frugivorous bats may have been a crucial selective advantage over insectivorous conspecifics with lower levels of antioxidants in their diet, and thus shorter life-spans. However, this hypothesis needs further confirmation, for example by studying the effect of diet on measures of oxidative stress within omnivorous bat species, and by comparing the levels of ROM and concentration of plasma antioxidants among bats with different life expectancies.

Conclusion

To conclude, we provide the first comprehensive study on measures of oxidative stress in free-ranging, long-lived mammals, providing evidence for a link between oxidative stress measures and dietary niches of bats. Our study sheds new light on the potential influence of an animal's ecological niche on proximate mechanisms of ageing. Furthermore, we showed that bats in general exhibit lower levels of ROM than non-flying mammals of similar body mass, which may account for the relatively longer life span of bats compared to rodents.

Acknowledgments

We are grateful to Daniel Lewanzik, Oliver Lindecke and Tobias Teige for their help in the field. We thank the Organization for Tropical Studies (OTS) for allowance to use their facilities and for providing logistic support and the Costa Rican authorities for granting permission to conduct this research [186-2012-SINAC]. This study has been funded by the Deutsche Forschungsgemeinschaft (DFG Vo 890/25).

References

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

Baker, R. J. and Clark, C. L. (1987) *Uroderma bilobatum*. *Mammalian Species* **279**, 1-4.

Balaban, R. S., Nemoto, S. and Finkel, T. (2005) Mitochondria, oxidants, and aging. *Cell* **120**, 483-495.

Barclay, R. M., Harder, L. D., Kunz, T. H. and Fenton, M. B. (2003) Life histories of bats: life in the slow lane. In: *Bat ecology*, 209-253.

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

Carocho, M. and Ferreira, I. C. F. R. (2013) A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds,. screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology* **51**, 15-25.

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

Cloutier, D. and Thomas, D. W. (1992) *Carollia perspicillata*. *Mammalian Species* **417**, 1-9.

Droege, W. (2002) Free radicals in the physiological control of cell function. *Physiological Reviews* **82**, 47-95.

Dumont, E. R., Kunz, T. H. and Fenton, M. B. (2003) Bats and fruit: an ecomorphological approach. In: *Bat ecology*, 398-429.

Garratt, M. and Brooks, R. C. (2012) Oxidative stress and condition-dependent sexual signals: more than just seeing red. *Proceedings of the Royal Society B* **279**, 3121-3130.

Goyns, M. H. (2002) Genes, telomeres and mammalian ageing. *Mechanisms of Ageing and Development* **123**, 791-799.

Grafen, A. (1989) The phylogenetic regression. *Philosophical Transactions of the Royal Society B* **326**, 119-157.

Harman, D. (1955) *Aging: a theory based on free radical and radiation chemistry*. University of California Radiation Laboratory.

Hoffmann, F. G. and Baker, R. J. (2003) Comparative phylogeography of short–tailed bats (Carollia: Phyllostomidae). *Molecular Ecology* **12**, 3403-3414.

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. *Journal of Health Science* **56**, 20-30.

Jansen, E. H. and Ruskovska, T. (2013) Comparative analysis of serum (anti)oxidative status parameters in healthy persons. *International Journal of Molecular Sciences* **14**, 6106-6115.

Jones, K. E., Purvis, A., Maclarnon, A. N. N., Bininda-Emonds, O. R. P. and Simmons, N. B. (2002) A phylogenetic supertree of the bats (Mammalia: Chiroptera). *Biological Reviews* **77**, 223-259.

Kwiecinski, G. G. (2006) *Phyllostomus discolor*. *Mammalian Species* **801**, 1-11.

LaVal, R. K. and Rodríguez, B. (2002) *Murcielagos de Costa Rica*. Editorial INBio.

Liochev, S. I. (2013) Reactive oxygen species and the free radical theory of aging. *Free Radical Biology and Medicine* **60**, 1-4.

MacArthur, R. H. (1967) *The theory of island biogeography*. Princeton University Press.

Maruoka, H. and Fujii, K. (2012) Effects of exercise and food consumption on the plasma oxidative stress. *Journal of Physical Therapy Science* **24**, 37-41.

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

Ortega, J. and Castro-Arellano, I. (2001) *Artibeus jamaicensis*. *Mammalian Species* **662**, 1-9.

Pagel, M. (1999) Inferring the historical patterns of biological evolution. *Nature* **401**, 877-884.

Paradis, E., Claude, J. and Strimmer, K. (2004) APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289-290.

Pearl, R. (1928) *The rate of living*. University of London Press.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. and R Core Team (2009) Nlme: Linear and nonlinear mixed effects models. R package version 3.1-96. R Foundation for Statistical Computing, Vienna, Austria

Price, T. (1997) Correlated evolution and independent contrasts. *Philosophical Transactions of the Royal Society B* **352**, 519-529.

R Development Core Team (2010) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org

Reth, M. (2002) Hydrogen peroxide as second messenger in lymphocyte activation. *Nature Immunology* **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? *International Journal of Biochemistry & Cell Biology* **34**, 1449-1460.

Sacher, G. A. Relation of lifespan to brain weight and body weight in mammals. *CIBA Foundation Colloquia on Aging* 5, 115-141. 1959.

Schinnerl, M., Aydinonat, D., Schwarzenberger, F. and Voigt, C. C. (2011) Hematological survey of common neotropical bat species from Costa Rica. *Journal of Zoo and Wildlife Medicine* **42**, 382-391.

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.

Selman, C., Blount, J. D., Nussey, D. H. and Speakman, J. R. (2012) Oxidative damage, ageing, and life-history evolution: where now? *Trends in Ecology & Evolution* **27**, 570-577.

Simmons, N. B. and Geisler, J. H. (1998) Phylogenetic relationships of Icaronycteris, Archaeonycteris, Hassianycteris, and Palaeochiropteryx to extant bat lineages, with comments on the evolution of echolocation and foraging strategies in Microchiroptera. *Bulletin of the AMNH* **235**.

Sohal, R. S. and Brunk, U. T. (1992) Mitochondrial production of pro-oxidants and cellular senescence. *Mutation Research/DNAging* **275**, 295-304.

Sohal, R. S., Svensson, I. and Brunk, U. T. (1990) Hydrogen peroxide production by liver mitochondria in different species. *Mechanisms of Ageing and Development* **53**, 209-215.

Sohal, R. S. and Weindruch, R. (1996) Oxidative stress, caloric restriction, and aging. *Science* **273**, 59-63.

Speakman, J. R. (2001) The evolution of flight and echolocation in bats: another leap in the dark. *Mammal Review* **31**, 111-130.

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.

Timm, R. M. and LaVal, R. K. (1998) A field key to the bats of Costa Rica. *Occasional Publication Series, University of Kansas Center of Latin American Studies* **22**, 1-30.

Timm, R. M. (1982) *Ectophylla alba*. *Mammalian Species* **166**, 1-4.

Timm, R. M. (1985) *Artibeus phaeotis*. *Mammalian Species* **235**, 1-6.

Turturro, A., Witt, W. W., Lewis, S., Hass, B. S., Lipman, R. D. and Hart, R. W. (1999) Growth curves and survival characteristics of the animals used in the Biomarkers of Aging Program. *The Journals of Gerontology Series A* **54**, B492-B501.

Voigt, C. C., Rex, K., Michener, R. H. and Speakman, J. R. (2008) Nutrient routing in omnivorous animals tracked by stable carbon isotopes in tissue and exhaled breath. *Oecologia* **157**, 31-40.

Wilhelm Filho, D., Althoff, S. L., Dafre, A. L. and Boveris, A. (2007) Antioxidant defenses, longevity and ecophysiology of South American bats. *Comparative Biochemistry and Physiology C* **146**, 214-220.

Wilkinson, G. S. and South, J. M. (2002) Life history, ecology and longevity in bats. *Aging Cell* **1**, 124-131.

York, H. A. and Billings, S. A. (2009) Stable-isotope analysis of diets of short-tailed fruit bats (Chiroptera: Phyllostomidae: Carollia). *Journal of Mammalogy* **90**, 1469-1477.

General Discussion

The general purpose of this thesis was to gain insights into eco-immunology and oxidative stress, two factors influencing survival and longevity in mammals. As Neotropical bats cover a wide variety of ecological niches and are surprisingly long-lived for their size, they are an ideal study system to investigate the link between aspects of the immune system and oxidative stress with life-history and ecological factors.

Eco-immunology of Neotropical bats

As eco-immunological studies on mammals so far concentrated on meta-analysis drawn from zoo records (Nunn et al. 2000; Nunn 2002; Nunn et al. 2003), studies on free-ranging populations are needed in order to understand the role of natural selection on eco-immunological aspects (Ricklefs and Wikelski 2002; Schulenburg et al. 2009). Bats may bear a high potential to answer eco-immunological questions, as they are amongst the most diverse mammalian clade with high variances in life-history traits that are likely to affect aspects of their immune system. Also, bats are known as reservoirs for various diseases (Calisher et al. 2006; Wibbelt et al. 2010; Wood et al. 2012), however, very little is known about their immune system.

Only recently, free-ranging bats have caught the attention of immunologists, and the newly emerging field of eco-immunology (Schulenburg et al. 2009) started to study aspects of the bat immune system (Schinnerl et al. 2011). However, only few studies have so far investigated specific aspects of the bat immune system and their connectivity with ecological factors. For example, Allen and colleagues (2009) found that the T-cell mediated immune response and bactericidal ability of the blood of female Brazilian free-tailed bats (*Tadarida brasiliensis*) vary among colonies and are potentially connected with differences in roosting ecology. Furthermore, Schinnerl and his colleagues (2011) found that measures of the cellular immune system vary greatly among Neotropical bat species that occupy numerous ecological niches. In previous studies on captive populations of carnivores and primates, diet has been speculated to be – among others – an important factor influencing the cellular immunity (Nunn 2002; Nunn et al. 2003). I therefore hypothesised that ecological factors such as dietary niche and roost site characteristics may be connected with aspects of the immune system, because they may promote specific pathogen transmission risks. In **Chapter I**, I investigated the mean total white blood cell (WBC) count and bacterial killing ability (BKA) of the plasma of 24 Neotropical bat

species. I found that BKA varies with roost types preferentially used by bat species. Plasma of bats that roost in ephemeral, less protected sites such as under modified leaves was more potent to kill *Escherichia coli* than plasma of species that roost in more permanent and protected sites such as hollow trees and abandoned buildings. These results are rather puzzling, as bats using permanent and protected roosts usually carry more ectoparasites than bats roosting in ephemeral sites (Patterson et al. 2007). Potentially, the relatively low humoral immunocompetence of the host rather than characteristics of the hosts' roost may determine the abundance of ectoparasites in bats. Also, species roosting in more ephemeral sites may have to switch roosts more often than species roosting in permanent sites and thus encounter a richer pathogenic environment, a theory that has found support in other mammals (Bordes et al. 2009).

Furthermore, I found that WBC count varied between bats according to their dietary niches, with insectivorous species having the lowest number of WBCs, followed by phytophageous and carnivorous bats. These findings go in line with the prediction that carnivorous species should have the highest disease transmission risk due to their relatively close relatedness to their prey. However, a previous study on captive carnivores did not find an association of WBC count with feeding ecology, e.g. with the proportion of meat in the diet (Nunn et al. 2003). Only eosinophils, which are cells that are important in fighting macroparasites (Delves et al. 2011), increased with the percentage of meat consumed. In my study, I also found eosinophils to be connected to diet, but as well were monocytes. Potentially, data drawn from individuals kept in captivity may not reflect the same selective constraints such as intensity of pathogenic environment found in natural populations. I conclude that, although analysis of data drawn from mammals in captivity may give a first insight in eco-immunological aspects, studies on wild populations are needed in order to understand how the immune system evolved to cope with the natural pathogenic environment an animal lives in.

Survival, longevity and the immune system

Especially when looking at life-history trade-offs, populations under natural selection need to be investigated, as in captivity, individuals may not face the same limitation in resources as under natural conditions. Mounting and maintaining immune functions is energetically costly (Lochmiller and Deerenberg 2000), and thus aspects of the immune system are candidates to be traded off against other life-history traits such as longevity (Zuk and Stoehr 2002). Longevity is especially important for animals with slow pace of life, because

prolonged survival may increase lifetime breeding success. On one hand, being able to mount a strong immune response may increase survival. For example, barn swallows (*Hirundo rustica*) that produce higher titres of antibodies against sheep red blood cells are more likely to survive until the next breeding seasons than individuals that produce low titres (Saino et al. 1997). On the other hand, 73% of eider ducks (*Somateria mollissima*) that have mounted a humoral immune response against three agents have been shown to disappear from their breeding site (Hanssen et al. 2004). Therefore, although being beneficial in the first place, mounting and maintaining immune functions may also have negative effects on survival.

Little is known about how aspects of the immune system correlate with longevity in mammals, with the exception of a few studies on laboratory organisms such as mice, where inhibition of anti-inflammatory cytokines reduces longevity (Belloni et al. 2010). Bats are an especially interesting mammalian group considering longevity, as they exhibit exceptionally long life-spans. They live on average 3.5 times as long as similar sized terrestrial mammals (Austad and Fischer 1991; Brunet-Rossinni 2004), and follow an extreme K-strategy with producing only one to two young per year. In **Chapter II**, I therefore asked if aspects of the immune system of bats are connected to survival and longevity, using the greater sac-winged bat (*Saccopteryx bilineata*) as model system. This species shows a high roost fidelity and thus, if individuals disappear from the roosting site, they most likely have died. I found that individuals with high WBC counts and high BKA of the plasma were less likely to be encountered in the colony half a year after sampling. Therefore, investing in cellular and humoral immune components may on one hand increase the individual's ability of coping with infections, but may on the other hand reduce the individual's overall probability to survive until the next breeding season. In fact, high white blood cell counts may indicate an acute infection, thus individuals with high counts may disappear from the colonies due to a lethal disease. I found immunoglobulin G titers to increase with increasing age, probably mirroring the number of infections an individual encountered during its life. In agreement with the finding that individuals with high WBC count disappear from the colony, I found older bats to have lower WBC counts than young ones. Thus, although being beneficial in the first place in order to fight parasites and pathogens, high investment in immune functions may have long-term disadvantages for an individual, such as promoting autoimmunity (Balomenos and Martinez 2000; Viau and Zouali 2005), and thus might to a certain extent be selected against.

Immune response and oxidative stress

The question arises what costs that are likely to reduce longevity come along with mounting and maintaining immune functions. One candidate is oxidative stress, i.e. an imbalance between reactive oxygen species (ROS) and antioxidants. ROS are produced during energy production in the mitochondria and their negative effect can be mitigated by either endogenous or exogenous (diet-derived) antioxidants. As ROS damage lipids, DNA and proteins (Sohal et al. 1990; Sohal and Brunk 1992; Goyns 2002), and the antioxidants barrier raised by an individual is not sufficient to fully mitigate these negative effects, the resulting oxidative stress causes senescence in cells and is therefore regarded as the main cause of ageing (Harman 1955).

During an immune response, higher amounts of ROS are produced since the metabolism of the host is usually elevated (Sheldon and Verhulst 1996), which leads to a higher mitochondrial activity. Consequently, sickness behaviour increases ROS production (Finkel and Holbrook 2000). Furthermore, white blood cell subtypes involved in cellular immune responses produce ROS to directly kill pathogens (Droege 2002), and to enhance the activation of T-lymphocytes (Droege 2002; Reth 2002). In **Chapter III**, I asked if mounting a cellular immune response leads to increased oxidative stress in long-lived bats. Due to their exceptional longevity, one would on one hand expect bats to show reduced signs of oxidative stress. On the other hand, as bats have especially high metabolic rates due to active flight, they may also produce more ROS. I found that short-tailed fruit bats (*Carollia perspicillata*) that showed a cellular immune reaction to injections with lipopolysaccharides (LPS) had elevated concentrations of reactive oxygen metabolites (ROM) in their plasma, which mirrors the total ROS produced. Antioxidant concentration in plasma was the same before and after injection with LPS. In the control group that was injected with phosphate buffered saline solution (PBS) only, I did not find a change in the concentration of ROM or antioxidants. Therefore, I showed that a cellular immune reaction increases measures of oxidative stress in bats, a finding that goes in line with several studies on birds (Costantini and Møller 2009). I conclude that although bats have relatively low levels of oxidative stress in general (Brunet-Rossinni 2004; Wilhelm Filho et al. 2007), they may still suffer from long-term consequences of elevated ROS production during a cellular immune response when facing acute or even chronic infections.

Oxidative stress and diet

It has previously been found that bats produce relatively low levels of pro-oxidants compared to similar-sized, terrestrial mammals (Brunet-Rossinni 2004; Wilhelm Filho et al. 2007). However, these studies were confined to single species and were rather inconsistent. In **Chapter IV**, I therefore aimed at providing a comprehensive dataset on measures of oxidative stress in bats, and asked furthermore, if ecological factors are connected with oxidative stress, in particular if the level of antioxidants may be largely influenced by ecological niche. Endogenously produced enzymatic and non-enzymatic antioxidants are not efficient enough to neutralise ROS to a sufficient extent, which is why dietary antioxidants play a major role in defending ROS. Feeding on diets with high antioxidant contents such as fruits has therefore been hypothesised to reduce oxidative stress. Studies on the beneficial effect of dietary antioxidants have been mainly confined to humans (reviewed in Carocho and Ferreira 2013) and birds (reviewed in Garratt and Brooks 2012), and are rather inconclusive. I found that bat species feeding exclusively on fruit have the lowest concentration of ROM in their plasma, followed by species feeding partly on fruits. Bats that did not include fruits in their diet had the highest level of ROM. I speculate that low levels of ROM may account to more antioxidants neutralising ROS, and that the evolutionary shift of bats from insectivory to a fruity diet may have been facilitated by the beneficial effect of these dietary antioxidants. It remains to be tested if bats with low levels of ROM in general live longer and thus if frugivorous species have an evolutionary advantage over non-frugivores. However, as the life expectancies of most Neotropical bat species are unknown, the dataset I provided in **Chapter IV** needs to be expanded by bats of the temperate zone with documented life-spans.

As I showed in **Chapter I**, dietary niche is also connected to the number of white blood cells. Therefore, an additional aspect to ROM being connected to dietary niche may be the ROS produced by immune cells, which might potentially lead to an increased concentration of ROM in plasma of species occupying niches with are connected to higher WBC counts. This hypothesis is supported by the findings of **Chapter III**, where I showed a significant correlation between WBC count and ROM. In **Chapter I**, I found insectivorous bats to have the lowest WBC count, which would in theory also lead to lowest production of ROS. However, in **Chapter IV**, I did not find insectivorous (non-frugivorous) bats to have the highest level of ROM in their plasma, and therefore I speculate that the production of ROS by immune cells may not *per se* lead to high levels of oxidative stress but that there are potentially many other factors involved.

Perspectives

Studies investigating the immune system and oxidative stress in mammals have so far been confined to laboratory species. The results of my thesis show that is it important to investigate populations under natural selection in order to find potential connections between aspects of the immune system and oxidative stress with ecological factors and life-history traits Therefore, studies on free-ranging populations are urgently needed in order to understand mechanisms of adaptation and the evolutionary ecology of mammals in general. Due to recent improvements and simplification of field-ready methods, eco-immunology is a promising topic and more studies on natural populations are to be expected.

While selected factors such as diet may only promote certain aspects of physiology such as those of the immune system and oxidative stress, many other factors may contribute and form a rather complex picture of what influences the evolution of physiological traits in mammals. For example, it remains to be investigated if social system is connected to the immune system in free-ranging mammals, a pattern that has previously been found in studies on captive populations (Nunn et al. 2000). Sexually transmitted diseases may be especially prominent under natural conditions, compared to animals kept in captivity where professional care-takers and veterinarians usually aim at reducing the risk of infection among individuals. Therefore, comparing aspects of the immune system among free-ranging mammals with contrasting social systems may shed light on the adaptability of the immune system to cope with infection risk e.g. due to frequent social contacts. Furthermore, in order to be able to distinguish between cause and consequence, experimental studies would be needed both to determine what factors influence aspects of the immune system as well as oxidative stress in mammals. Thus, insights gained from laboratory species should be adapted to natural populations where possible in order to gain further knowledge on causative relationships between the ecology of an animal and its physiological characteristic. For example, one might conduct feeding experiments on omnivorous species temporarily kept in captivity, providing one group with naturally antioxidant rich food such as fruits, while the other groups is fed on a diet with relatively low antioxidants content. However, experimental studies on free-ranging mammals are difficult to conduct. Furthermore, not all ecological factors can be experimentally manipulated, and therefore, correlative studies such as the ones presented in this thesis may give a first idea on potential evolutionary trade-offs in natural populations. Finding that the immune system and oxidative stress are connected to the ecology and life-history of bats

therefore gives a first idea on what factors may determine important physiological traits in free-ranging mammals.

References

Allen, L. C., Turmelle, A. S., Mendoca, 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*). *Journal of Comparative Physiology B* **179**, 315-323.

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

Balomenos, D. and Martinez, A. (2000) Cell-cycle regulation in immunity, tolerance and autoimmunity. *Immunology Today* **21**, 551-555.

Belloni, V., Faivre, B., Guerreiro, R., Arnoux, E., Bellenger, J. and Sorci, G. (2010) Suppressing an anti-inflammatory cytokine reveals a strong age-dependent survival cost in mice. *Plos One* **5**, e12940.

Bordes, F., Morand, S., Kelt, D. A. and Van Vuren, D. H. (2009) Home range and parasite diversity in mammals. *The American Naturalist* **173**, 467-474.

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

Calisher, C. H., Childs, J. E., Field, H. E., Holmes, K. V. and Schountz, T. (2006) Bats: important reservoir hosts of emerging viruses. *Clinical Microbiology Reviews* **19**, 531-545.

Carocho, M. and Ferreira, I. C. F. R. (2013) A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds,. screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology* **51**, 15-25.

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

Delves, P. J., Martin, S. J., Burton, D. R., and Roitt, I. M. (2011) *Roitt's essential immunology*. Wiley.

Droege, W. (2002) Free radicals in the physiological control of cell function. *Physiological Reviews* **82**, 47-95.

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

Garratt, M. and Brooks, R. C. (2012) Oxidative stress and condition-dependent sexual signals: more than just seeing red. *Proceedings of the Royal Society B* **279**, 3121-3130.

Goyns, M. H. (2002) Genes, telomeres and mammalian ageing. *Mechanisms of Ageing and Development* **123**, 791-799.

Hanssen, S. A., Hasselquist, D., Folstad, I. and Erikstad, K. E. (2004) Costs of immunity: immune responsiveness reduces survival in a vertebrate. *Proceedings of the Royal Society B* **271**, 925-930.

Harman, D. (1955) *Aging: a theory based on free radical and radiation chemistry*. University of California Radiation Laboratory.

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

Nunn, C. L. (2002) A comparative study of leukocyte counts and disease risk in primates. *Evolution* **56**, 177-190.

Nunn, C. L., Gittleman, J. L. and Antonovics, J. (2000) Promiscuity and the primate immune system. *Science* **290**, 1168-1170.

Nunn, C. L., Gittleman, J. L. and Antonovics, J. (2003) A comparative study of white blood cell counts and disease risk in carnivores. *Proceedings of the Royal Society B* **270**, 347-356.

Patterson, B. D., Dick, C. W. and Dittmar, K. (2007) Roosting habits of bats affect their parasitism by bat flies (Diptera: Streblidae). *Journal of Tropical Ecology* **23**, 177-189.

Reth, M. (2002) Hydrogen peroxide as second messenger in lymphocyte activation. *Nature Immunology* **3**, 1129-1134.

Ricklefs, R. E. and Wikelski, M. (2002) The physiology/life-history nexus. *Trends in Ecology & Evolution* **17**, 462-468.

Saino, N., Bolzern, A. M. and Møller, A. P. (1997) Immunocompetence, ornamentation, and viability of male barn swallows (*Hirundo rustica*). *Proceedings of the National Academy of Sciences* **94**, 549.

Schinnerl, M., Aydinonat, D., Schwarzenberger, F. and Voigt, C. C. (2011) Hematological survey of common neotropical bat species from Costa Rica. *Journal of Zoo and Wildlife Medicine* **42**, 382-391.

Schulenburg, H., Kurtz, J., Moret, Y. and Siva-Jothy, M. T. (2009) Introduction. Ecological immunology. *Philosophical Transactions of the Royal Society B* **364**, 3-14.

Sheldon, B. C. and Verhulst, S. (1996) Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution* **11**, 317-321.

Sohal, R. S. and Brunk, U. T. (1992) Mitochondrial production of pro-oxidants and cellular senescence. *Mutation Research/DNAging* **275**, 295-304.

Sohal, R. S., Svensson, I. and Brunk, U. T. (1990) Hydrogen peroxide production by liver mitochondria in different species. *Mechanisms of Ageing and Development* **53**, 209-215.

Viau, M. and Zouali, M. (2005) B-lymphocytes, innate immunity, and autoimmunity. *Clinical Immunology* **114**, 17-26.

Wibbelt, G., Moore, M. S., Schountz, T. and Voigt, C. C. (2010) Emerging diseases in Chiroptera: why bats? *Biology Letters* **6**, 438-440.

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., Peel, A. J., Kamins, A. O., Fahr, J., Ntiamoa-Baidu, Y., Suu-Ire, R., Breiman, R. F., Epstein, J. H., Field, H. E. and Cunningham, A. A. (2012) A framework for the study of zoonotic disease emergence and its drivers: spillover of bat pathogens as a case study. *Philosophical Transactions of the Royal Society B* **367**, 2881-2892.

Zuk, M. and Stoehr, A. M. (2002) Immune defense and host life history. *American Naturalist* **160**, S9-S22.

Acknowledgement

I am deeply thankful for the tremendous help and support I received during the last few years, and although words can hardly express my gratefulness, I would like to use the following lines to thank the most important people:

First of all, I would like to express my deepest gratitude to my supervisor PD. Dr. Christian Voigt, who introduced me to bats as fantastic model organisms, sharing his knowledge patiently, and who has always been an incredibly supportive mentor. I would also like to especially thank him for giving me the opportunity to develop my own research ideas and work in probably one of the most amazing places on earth, the Caribbean lowland rainforest in Costa Rica.

I am very grateful to my collaborator Dr. Gábor Czirják, who introduced me to eco-immunology and taught me various immunological techniques. Also, I am very thankful for all the prolific and interesting discussions and helpful advices he gave me at various stages of my thesis.

I would like to thank Prof. Dr. Heribert Hofer for giving me the opportunity to work in such a stimulating environment at the Leibniz Institute for Zoo and Wildlife Research (IZW) and for the support he provided during my thesis.

Katja Pohle has been very helpful with various kinds of immunological laboratory analysis and helped me out on so many occasions when I was short in material. I would also like to thank Dr. Alexandre Courtiol for statistical advice.

My special thanks go to the Bat Research Group, the Department of Evolutionary Ecology and the doctoral students of the IZW for the many fruitful discussions and helpful hints during various stages of my thesis. I would like to specially thank Tanja Noventa, Olga Heim, Daniel Lewanzik, David Lehmann, Silke Voigt-Heucke, Sebastian Menke, Alexandre Courtiol, Oliver Lindecke, Simon Ghanem, Sara Troxell, Sonja Heinrich, Milena Stillfried, Janine Helms, Sven Kühlmann, Johanna Painer, Linn Lehnert, Anne Seltmann, Christoph Kurze, Saskia Wutke, Manuela Fischer, Matze Meier and Mark Gillingham. I am deeply thankful for having such great friends, with whom I could share great moments and who provided me with so much emotional support during times of frustration.

I am very grateful to Doris Fichte, Anja Luckner, Karin Sörgel, Dagmar Boras and Katrin Hohenstädter, who very patiently helped me with administrative issues.

I thank OTS for allowing me to conduct research on their property and the Costa Rican authorities (SINAC/MINAE) for permission to perform these studies. I would also like to thank the staff of La Selva Biological Station that made every stay comfortable, especially Carlos de la Rosa, Ronald Vargas, Bérnal Matarrita and Danilo Bernes. I am deeply thankful for all the friends I gained during my stays in Costa Rica. Christine Creese, Virginia Weigand-Noble, Kelsey Reider, Kelly Kuhn, Jenny Stynoski, Mike Wells, Andrew Iain Bruce, Carlos Garcia-Robledo, Susan Whitehead, Ralph Simon, Paula and Merlin Tuttle, Gloriana Chaverri, Diego and Rae "Ping" Dierick, Justin Nowakowski, Kim and John Smiley, Claudia Nocke, Carissa Ganong and Meghan Fitzgerald, thank you so much for all the wonderful moments at La Selva, the great discussions at lunchtime, the coffee breaks, ice cream on the bridge and art nights. I would also especially like to thank Silke Voigt-Heucke, Ivailo Borrisov, Daniel Lewanzik, Oliver Lindecke and Tobias Teige for help in the field and for great company during long and exciting nights in the jungle.

My gratitude goes to the German Research Fondation (DFG) who kindly provided financial support for the oxidative stress studies.

Sara Troxell, Daniel Lewanzik, Simon Ghanem, Sebastian Menke, Julia Schad and Olga Heim gave helpful comments on early drafts of the manuscripts and helped to substantially improve them.

I would also like to thank all the other helping and supporting hands whose names did not find their way into this thesis.

Finally, but most importantly, I would like to thank my family, especially my parents Stefan and Rosmarie Schneeberger and my siblings Remo and Bettina. Thank you so much for your endless support and encouragement throughout my life, and for giving me the freedom to follow my dreams.

Thank you!

Karin Schneeberger

Curriculum Vitae

Der Lebenslauf ist in der Online-Version
aus Gründen des Datenschutzes nicht enthalten

Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Doktorarbeit eigenständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Berlin, 20.08.2013

Karin Schneeberger