Mammalian Biology 80 (2015) 312–315

Contents lists available at ScienceDirect

Mammalian Biology

journal homepage: www.elsevier.com/locate/mambio

Short Communication

No short-term effect of handling and capture stress on immune responses of bats assessed by bacterial killing assay

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ARTICLE INFO

Article history:
Received 5 December 2014
Accepted 19 February 2015
Handled by Danilo Russo
Available online 27 February 2015

Keywords:
Acute stress
Bacterial killing assay
Constitutive immunity
Innate immune system
Chiroptera

ABSTRACT

Ecoimmunology of wild animals becomes increasingly important. However, there are methodical limitations, especially when working on small mammals, e.g. small sample volume and acute stress associated with capture, handling and sampling that can influence immune parameters. The plasma bacterial killing assay measures innate humoral immune responses, mainly complement activity. It is a powerful tool with many methodical advantages. To avoid investigation of artefacts in future ecoimmunological studies the influence of acute stress on the bacterial killing activity was assessed.

Bats (*Nyctalus noctula*, $n = 9$) were repeatedly sampled in three time intervals up to 97 min after capture. Bacterial killing activity against *Escherichia coli* was measured using a microplate absorbance reader. Bacterial killing activity was not influenced by capture, handling and sampling. Hence, released stress hormones did not affect circulating complement activity. To conclude, the plasma bacterial killing assay is reliable and efficient ecoimmunological tool in wildlife studies even of small mammals.

Ecoimmunology is a relatively new field of research with a considerable increase in interest. It explains temporal and geographical variations of infectious diseases by understanding variability of the hosts' immune responses in ecological and environmental contexts (Boughton et al., 2011), which lead to a varying disease susceptibility (Demas and Nelson, 2012). Immunological studies on wild animals are difficult to conduct since the blood volume is limited and the collection time includes capturing, handling, and sampling of animals. These procedures are unknown and potentially life-threatening situations for the wild animal and thus cause acute stress (Grandin, 1997; Widmaier et al., 1994).

Under acute stress glucocorticoid concentration increases within a few minutes after first contact with the stressor (Romero and Reed, 2005). Stress modulated immune responses depend on life history, e.g. reproduction and are highly flexible (Martin, 2009). In contrast to the general assumption that acute stress fortifies immune function (Dhabhar, 2002; Martin, 2009), in some birds handling duration and immune function of blood components are negatively correlated (Matson et al., 2006).

A powerful tool in ecoimmunology is the "bacterial killing assay" (BKA). This assay assesses the ability of whole blood or plasma to kill bacteria. A defined number of bacteria is added to the sample and bacteria growth is quantified after incubation using a microplate reader. Bacterial killing ability of the samples is determined relative to a positive control (everything without sample). It has a broad range of applications on vertebrates (French and Neuman-Lee, 2012; Liebl and Martin, 2009; Matson et al., 2006).

Depending on the blood sample type and bacteria strain various immune parameters can be measured (French and Neuman-Lee, 2012; Pilosof et al., 2014). Using whole blood, a mixture of components of humoral (Merchant et al., 2003) and cellular innate immunity (Keusch et al., 1975) are assessed. Plasma samples provide information only on components of the humoral innate immunity that are involved in killing bacteria. Engaged components are largely antibodies and proteins of the complement system (French and Neuman-Lee, 2012; Millet et al., 2007). Presupposing the same methodology BKA can be compared between studies and taxa as the activity is determined by species-independent reagents. Further advantages of this assay are small sample volumes, use of easy-storable plasma samples, short analysis time, and high adaptability to scientific questions (French and Neuman-Lee, 2012; Millet et al., 2007). These characteristics make the BKA an excellent tool to study the health condition of populations in an ecological or evolutionary context. Furthermore, the impact of external harmful

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http://dx.doi.org/10.1016/j.mambio.2015.02.005
1616-5047/

influences (e.g. sewage contamination, Pilosof et al., 2014) on the immunity of wild animals can be determined. Therefore, an assessment of performance and optimisation of this assay is essential for ecoimmunological studies.

Plasma BKA measures humoral innate immune components which can be influenced by acute stress (Sapolsky et al., 2000). The question arises whether this useful tool of BKA can be reliably used in the field as capture, handling and blood sampling cause acute stress in wild animals. To avoid investigation artefacts in ecoimmunological studies, it is important to assess the influence of stress on BKA.

Bats and rodents are ideal model organisms for ecoimmunological studies as many of them are highly endangered and important reservoirs for many zoonotic viruses (Luis et al., 2013). Therefore, this study assessed if BKA is influenced by acute stress in bats.

Nyctalus noctula (Schreber, 1774) is a common large insectivorous bat (IUCN: least concern (Csorba et al., 2008); forearm length: 47.3–58.9 mm, body mass: 21–30 g (Dietz et al., 2009), which is distributed over most of Europe and in some parts of Asia (Csorba et al., 2008). This migrating species is a reservoir host of many virus groups like paramyxovirus (Kurth et al., 2012), coronavirus (Reusken et al., 2010), and herpesvirus (Wibbelt et al., 2007).

Reproductive ($n = 6$, 31st August: spermatogenesis, mating) and non-reproductive ($n = 3$, 30th April: sexual resting time) male individuals of *N. noctula* (Racey, 1974) were collected from bat boxes within a small deciduous forest in Hesse, Germany (50°33′44″ N, 8°37′34″ E; 191 m.a.s.l.). Species, sex, and reproductive state (extension of the epididymides (Encarnação et al., 2004)) were visually determined. Body mass (Kern & Sohn GmbH, Balingen, Germany; accuracy 0.01 g) and forearm length (callipers; Hydrotec Technologies, Wildeshausen, Germany; accuracy 0.01 mm) were measured.

Blood samples ($n = 26$) were collected by venipuncture using a lancet to pierce the propatagiale vein. Blood was collected in coated heparin-lithium microcapillary tubes (Sarstedt, Nümbrecht, Germany) until 20 μL of whole blood accumulated. Samples were collected three times per bat (one individual was only sampled twice) in time intervals (sampling point after capture 1: 3–10 min, 2: 27–44 min, 3: 56–68 min and 4: 97 min). Samples were kept on ice for less than 1 h until they were centrifuged and the plasma was collected and frozen at −20 °C until further analysis (maximal three weeks).

Bacterial killing assay was conducted after French and Neuman-Lee (2012). The microbes *Escherichia coli* (ATCC #8739, Epower; Doenitz ProLab, Augsburg, Germany) were reconstituted in 0.9% Phosphate Buffered Solution (PBS) following manufacturer instructions. This Gram-negative strain of bacteria is highly susceptible to the killing activities of blood and is mainly killed by humoral components (Merchant et al., 2003; Millet et al., 2007). Bacterial stock was diluted to a working concentration of 10^5 bacteria/mL. In 96-well microplates (Roth, Karlsruhe, Germany) 2 μL of plasma samples were diluted 1:8 in PBS. To each well, 6 μL of the bacteria working solution were added. After incubation, Tryptic Soy Broth (Sigma–Aldrich, Taufkirchen, Germany) was added and background absorbance was measured at 300 nm (Infinite M200; Tecan, Crailsheim, Germany). The plates were incubated at 37 °C for 12 h and the absorbance was measured again. All plates were run with positive (all components without plasma) and negative controls (all components without bacteria). To determine plasma BKA, the percent of killed bacteria relative to positive control was calculated with following formula:

$$\mathrm{BKA} = 1 - \left(\frac{\text{absorbance sample} - \text{background absorbance sample}}{\text{absorbance positive controls} - \text{background absorbance positive controls}} \right) \times 100 \quad (1)$$

Table 1
Nested GLM with explanatory factors on BKA of *Nyctalus noctula*.

Explanatory factor	SS	*P*
Individual	477.20	0.014
Time of handling (Individual)	155.78	0.271
Error	88.72	–
Model R^2	0.938	–
Model *P*	–	0.004

Change in BKA (ΔBKA) per individual was calculated as BKA of sampling point 1 subtracted from BKA of sampling point 2, 3, or 4, of the respective individual.

To examine variations in plasma BKA of one individual at different sampling points, a nested general linear model (GLM) with the explanatory factors "individual" and "time of handling" was built (Statistica 10.1, StatSoft Inc., 13.0). As a measure of the impact of each predictor, the sums of squares (SS) were calculated.

Acute stress did not influence plasma BKA as the time of handling had no influence on BKA ($P = 0.300$) within a maximum of 97 min for *Nyctalus noctula* (GLM, $R^2 = 0.938$; df = 17; $P = 0.004$) (Table 1, Fig. 1). Bacterial killing activity differed, however, significantly between individuals ($P = 0.014$).

Sampling under three minutes is standard to get baseline values for hormone measurements (Romero and Reed, 2005). Stress hormone levels, however, increase more rapidly than immune responses (Buehler et al. 2008). While a sample under three minutes indicate almost baseline levels for stress hormones (Romero and Reed, 2005), in measurements of immune responses a change can be observed in samples taken after 20–30 min (Buehler et al. 2008). For Chiroptera, handling and capture procedures trigger the release of stress hormones after 15 min (Reeder et al., 2004; Widmaier and Kunz, 1993). Therefore, the chosen timeline gives accurate results on the influence of acute stress on BKA measured in plasma.

The ability of bats to activate complement proteins and the concentration of circulating levels of proteins was not affected by handling, capture and physical stress due to previous blood sampling. Hence, stress hormones did not influence BKA in *N. noctula*. In contrast, a decrease in BKA with time after capture was observed in several bird species (Matson et al. 2006). It can be hypothesised, that the impact of acute stress on humoral innate immune responses is very complex and species-specific as physiological responses to stress depend on species (Korte et al. 2004) and may even reflect ecological differences between species (Buehler et al. 2008).

Maintaining the complement activity during stress has severe health implications as it is an important first line defence and a chief component of the immune system (Ricklin et al., 2010). Individuals have to balance benefits and risks of immune modulation (Lochmiller and Deerenberg, 2000). During an acute stress event, energy-costly "fight-or-flight" behaviours increase risk of wounds and therefore the risk of infections (Segerstrom, 2007). Complement proteins prevent infections by microbes that penetrate through wounds. Metabolic costs of the complement system are relatively low compared to other immune responses (e.g. induced cell-mediated responses) (Lee, 2006). An infection caused by penetrating microbes would result in mounting energetically costly inflammatory immune responses (Derting and Compton, 2003; Janeway et al., 2001). Hence, maintenance of the complement activity during acute stress prevents infections and allows a reallocation