



Research

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No fever and leucocytosis in response to a lipopolysaccharide challenge in an insectivorous bat

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Bat immune systems may allow them to respond to zoonotic agents more efficiently than other mammals. As the first line of defence, the taxonomically conserved acute phase immune reaction of leucocytosis and fever is crucial for coping with infections, but it is unknown if this response is a key constituent to bat immunological success. We investigated the acute phase reaction to a standard lipopolysaccharide (LPS) challenge in Pallas's mastiff bats (*Molossus molossus*). Challenged bats lost mass, but in contrast to other mammals showed no leucocytosis or fever. There also was no influence on body temperature reduction during torpor. When compared to recent genome-wide assays for constituent immune genes, this lack of a conserved fever response to LPS contributes to a clearer understanding of the innate immune system in bat species and of the coevolution of bats with a wide diversity of pathogens.

1. Introduction

Bats host more zoonotic virus species than other mammalian orders, largely without presenting incidence of disease [1,2]. This renders bats potential reservoir hosts and relevant research has focused on specific antibody responses to pathogens [1–3]. The less-investigated first line of defence of the immune response, the acute phase reaction, contributes to the early control of infections and may have influenced bat coevolution with a wide diversity of pathogens that are highly virulent in other species. What little is known about bat acute phase response suggests that they are no different than other mammals [1,4] although there may be novel adaptations to successfully cope with infections [3,4].

Increases in body temperature (T_B , fever), circulating white blood cells (WBC, leucocytosis) and mass loss are common parts of the conserved acute phase reaction and can be induced experimentally [5–7]. Fever is especially important in combating infections by inhibiting viral and bacterial replication as well as enhancing leucocyte responses to infection [6]. This metabolic and temperature cycling occurs in bats on a daily basis as they transition from resting states to T_B greater than 40°C during flight, which is hypothesized to mimic daily fever [3]. At the other end of the spectrum, many bats lower T_B and metabolic rate on a daily basis through torpor and seasonally through hibernation. Such metabolic suppression can have broad effects on immune function. For example, during hibernation, fever response is delayed in ground squirrels until they return to normothermic temperatures [8], and immune challenges prevent full suppression of T_B during torpor in grey mouse lemurs [9]. To test the acute phase responses in Pallas's mastiff bats (*Molossus molossus*), we used a standard acute phase immune challenge

designed to elicit both fever and leucocytosis. *Molossus molossus* use torpor as a daily energy-saving strategy [10], and we hypothesized that if bat acute phase immune reactions are concordant with mammalian patterns, lipopolysaccharide (LPS) injection should reduce daily temperature fluctuations [9], and/or that daily return to normothermy should trigger a delayed fever response [8]. Furthermore, we hypothesized that LPS should induce the standard responses of mass loss and leucocytosis.

2. Material and methods

We captured bats at evening emergence from roosts in Gamboa, Panama (9°07' N, 79°42' W; 2008–2014). We recorded sex, reproductive status, mass and age from all individuals and produced blood smears from a drop of blood collected in a heparin-coated capillary tube [11]. Most individuals ($n = 60$) were then released within 2 h of capture.

We kept six females and seven males for experiments and housed them singly in boxes (20 × 15 × 6 cm) at ambient temperature ($\pm 28^\circ\text{C}$) and light. They received *ad libitum* water and were hand fed each evening with *ca* 2 g of mealworms and cat food (Whiskas, Germany). Subcutaneous thermo-sensitive PIT tags (0.1°C, Biothermo13, Biomark, USA) were used to monitor T_B . We injected 50 μl of 1 mg ml⁻¹ (4.53 ± 0.66 mg kg⁻¹) LPS (L2630, Sigma-Aldrich, Germany) in phosphate-buffered saline (PBS), or 50 μl of PBS only under the dorsal skin. LPS is a bacterial endotoxin and induces both fever and leucocytosis in mice and humans [5,7]. LPS and PBS were injected sequentially in random order (first LPS/second PBS or vice versa) with a minimum 24-h recovery period between treatments. T_B was recorded once per hour for 26–28 h post-injection. Females in this experimental group received the second treatment after 72 h, but we observed no difference in T_B after 24, 48 or 72 h, confirming that the 24-h recovery period was appropriate. We measured body mass and produced blood smears before and after each treatment. An additional 10 males and 11 females received the same challenge experiment, but no PIT tags. We estimated WBC numbers by counting leucocytes in 30 visual fields within the exterior monolayer of each smear at 125× magnification. We analysed the effect of the LPS and PBS treatments in R 3.0.2 [12] using a zero inflated negative binomial mixed effects model in *glmmADMB* with animal identity as a random effect and evaluated fixed factors using a likelihood ratio test (χ^2). To verify the inflammatory potential of our LPS, we injected six Seba's short-tailed bats (*Carollia perspicillata*) using the same experimental set-up and the WBC response was consistent with previous work [13]. Electronic supplementary material, tables S1 and S2, provide all data and counts from individual bats.

3. Results

T_B of captive bats ranged from 25.5 to 38.6°C and followed a pattern of lowered T_B during the day (torpor) and higher T_B at the beginning and end of the night (figure 1). T_B of experimentally challenged animals was the same as in the control at all times except during the initial handling/feeding (hour 0, figure 1; electronic supplementary material, table S3). There were no sex differences in T_B response (electronic supplementary material, table S3). Initial baseline WBC counts were low ($n = 94$; mean = 0.85 ± 0.08) and there was no effect of LPS ($\chi^2_1 = 1.30$, $p = 0.25$) or PBS ($\chi^2_1 = 2.37$, $p = 0.12$) injection on WBC counts (figure 2a). Bats injected with LPS lost more mass 24-h post-injection (LPS: mean = 0.55 ± 0.07 g; PBS: mean = 0.21 g \pm 0.06 g; $t_{32} = 3.45$, $p = 0.002$, figure 2b), again with no sex difference (LPS: $t_{31} = 0.20$, $p = 0.84$; PBS: $t_{31} = 0.15$, $p = 0.15$).

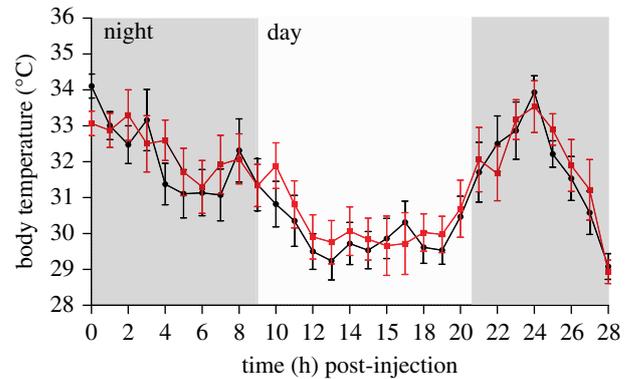


Figure 1. Body temperature (mean \pm s.e.) of bats 0–28 h post LPS (red, squares) and PBS (black, circles) injection. (Online version in colour.)

4. Discussion

Bats lost mass when challenged with LPS, but in contrast to other mammals [5,7], we found no fever. Also in contrast to other mammals, including another bat species, we found no leucocytosis [5,13]. Metabolic costs of an immune challenge are high and mass loss indicates mobilization of energy reserves to support the immune response [6]. Mass loss of 7% within the first 24 h of LPS injection is similar to studies in other small mammals (e.g. [7]).

More interesting is the indication that fever may not be part of *M. molossus* acute immune response, which warrants investigation in additional species. There is a trade-off between gene families that mitigate the effects of flight, particularly DNA damage sensors, and aspects of the immune system in bats [4]. This includes the loss of genes associated with sensing microbial DNA (e.g. AIM2 and IFI16 [4]), which may reduce bat sensitivity to bacterial challenges. Toll-like receptor 4 (TLR4) induces fever [6] downstream of CD14-LPS interactions [14], and TLR4 sequences are conserved between a fruit bat and other mammals [15]. If this is also true for *M. molossus*, activation of TLR4 through LPS does not result in fever response. It is possible that, as like in rodents, the extracellular domain of TLR4 is under varying selection [16], but that this selection has resulted in lowered affinity for ligands generated through CD14-LPS, and subsequent haplotypes of TLRs in bats resulted in an immune response that has eliminated or reduced fever [17]. It has been hypothesized that fluctuations in T_B in excess of 40°C during flight mimics fever in bats and allow for both flexible immune response and the ability to eliminate free radicals generated during flight [1,3,4]. Such adaptations to flight might mitigate the need for fever response through daily high T_B . However, birds also generate high T_B during flight but do show fever response to LPS [18]. Further work characterizing and quantifying the mechanisms that bats use to respond to various microbial classes is needed, particularly those related to negative selection on fever.

The lack of leucocytosis in *M. molossus* is unusual. Leucocytosis is a generalized response in vertebrates, and we confirmed the potency of our reagents in a different bat species (electronic supplementary material, tables S1 and S2, figure S1). Baseline circulating WBC in *M. molossus* were low, even compared to similarly sized bat species (three to four WBC per visual field at 200× [11]). Low T_B during seasonal hibernation reduces the number of circulating leucocytes in other mammals [19], but *M. molossus* reduces

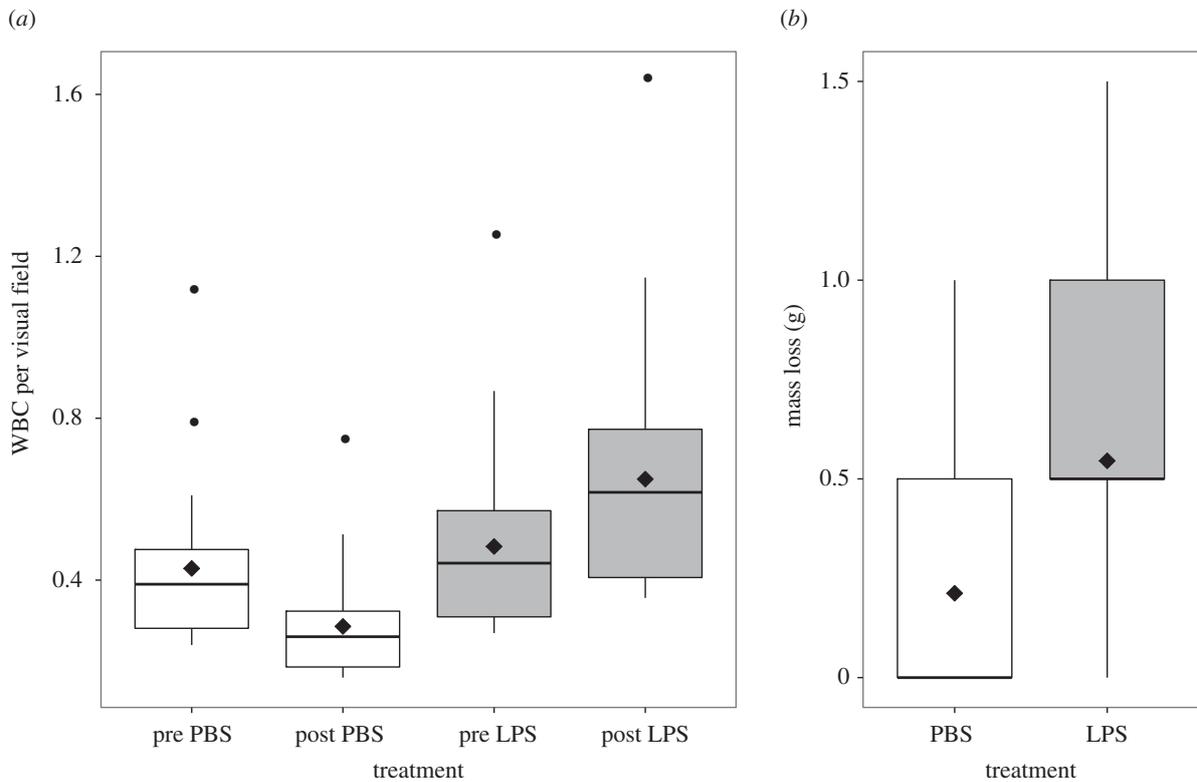


Figure 2. (a) No elevated WBC counts per 10 visual fields 24 h post PBS (white) or LPS (grey). (b) Bats lose more mass when injected with LPS than PBS. Box plots show the interquartile ranges, the median (line) and mean (diamond).

T_B daily to 28°C, and continues to do so after LPS challenge. Initial blood samples were collected from bats caught at evening emergence and held for at least 30 min before sampling. This stress should increase reactivity to LPS [20] if these bats were to mount a response. Further exploration is needed, particularly in bat species that use a broader range of T_B than that possible in the tropics.

The extent to which bat immune systems differ from other mammals remains unclear. So far, hypotheses that link bat immunology to the evolution of flight best explain genome-level immune adaptations. This includes mitochondrial adaptations to metabolic stress and reduction of free radicals [1], increased metabolic rates and body temperatures [3], and trade-offs with immune gene families [4]. Direct tests of the constituent immune responses, however, are necessary to identify the unusual ways in which these adaptations are truly employed [1,3,4]. Our results show that the conserved immune response of fever may no longer be intact [6], which would not be predicted from genomes that indicate

intact response pathways [4]. We are beginning to understand the complexity of bat adaptations to infection from genome to basic responses, but bat immunology is still in its infancy.

Ethics. Experiments were approved by ANAM, Panama (SE/A-112-13; SE/A-73-14) and the IACUC of the Smithsonian Tropical Research Institute (2012-0505-2015; 2014-0701-2017).

Data accessibility. The dataset supporting this article has been uploaded as part of the electronic supplementary material.

Authors' contributions. S.S. and M.T.O. carried out the experiments, statistical analysis and wrote the manuscript. D.K.N.D. and R.A.P. helped design the study and wrote the manuscript. All authors gave final approval for publication.

Competing interests. The authors have no competing interests.

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