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# Effect of sex and reproductive status on the immunity of the temperate bat *Myotis daubentonii*

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ABSTRACT

Studies of immunity in bat species are rare. However, it is important to determine immunological variations to identify factors influencing the health status of these endangered mammals from an evolutionary, ecological, conservation, and public health point of view. Immunity is highly variable and can be influenced by both internal (e.g. hormone levels, energy demand) and external factors (e.g. pathogens, climate). As bats have some peculiar ecological, energetic, and putative immunological characteristics, they are outstanding study organisms for ecoimmunological studies. We tested if (i) female bats have a higher immunity than males similar to most other mammalian species and (ii) individuals differ according to their energy demand (e.g. reproductive status). To study these questions, we sampled female and male *Myotis daubentonii* with different reproductive states and estimated their bacterial killing activity, hemolysis/hemagglutination titer, immunoglobulin G (IgG) concentration, and total and differential white blood cell counts. These methods characterize the cellular and humoral branches of both the adaptive and the innate immune responses of these individuals. Reproductively active males had lower cellular immunity compared to non-reproductive individuals. Pregnant females had increased IgG concentrations while hemolysis was enhanced during lactation. No clear trade-off between immunity and reproduction was found; instead immunity of males and female bats seems to be modulated differently due to varying hormonal and energetic states. Our data suggest that both adaptive and innate immunity as well as individual differences (i.e. sex and reproductive state) need to be considered to get a comprehensive overall picture of immunity in wild mammals.


## Introduction

Sex differences in immunity have been described for many species (Alexander and Stimson, 1988; Schuurs and Verheul, 1990). In general, males exhibit lower immune responses and thus are more susceptible to pathogens compared to females (Alexander and Stimson, 1988; Klein, 2000; Zuk and McKean, 1996). For example, males typically have lower immunoglobulin levels than females (Butterworth et al., 1967; Schuurs and Verheul, 1990). These differences have been mainly attributed to the differential effects of sex steroid hormones (e.g. testosterone) on the immune system (Da Silva, 1999). Besides hormones, energetic demands of individuals can also explain sex differences in immunity (Love et al., 2008). The energy budget of an individual is limited and must be allocated to all competing life-history traits (Demas et al., 2012; Ellison, 2003; Lochmiller and Deerenberg, 2000). The immune system is energetically costly in development, maintenance, and usage (Lochmiller and Deerenberg, 2000; Zuk and Stoehr, 2002). This leads to a trade-off with other costly life history traits such as reproduction and growth (French et al., 2007; Martin et al., 2008). Pregnancy and lactation are the most energetically costly phases of reproduction for most mammals (Hammond and Diamond, 1997; Thompson, 1993). During these energy demanding periods less energy seems to be available for immune functions as compared to non-reproductive stages (Moore and Hopkins, 2009; Nelson et al., 2002; Speakman, 2008). For instance, Siberian hamsters (*Phodopus sungorus*) have reduced immunoglobulin production during pregnancy and lactation as a result of energy reallocation to reproduction (Drazen et al., 2003).

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Northern temperate-zone bats are outstanding organisms to study variations in immune function. They demonstrate a unique combination of life-history traits and energetic features (Barclay and Harder, 2003; Becker et al., 2013a). The energy demand of temperate bats fluctuates during seasons and differs between sexes according to the asynchronous reproductive periods of males and females (Barclay, 1991; Becker et al., 2013b; Encarnação and Dietz, 2006). Males are reproductively active during spermatogenesis in midsummer with a peak in August (Encarnação and Dietz, 2006; Encarnação et al., 2004). For female bats, the reproductively active period starts with pregnancy after fertilisation in spring. Lactation begins in midsummer and post-lactation in late summer (Dietz et al., 2009). In addition to the energetic investment into reproduction, it is highly important to maintain immune function during these periods as reproductive output can be reduced during an infection (Telfer et al., 2005). Bats can cover such high energy requirements by increasing fat reserves (Encarnação et al., 2004; Kunz et al., 1998) or food intake (e.g. Anthony and Kunz, 1977; Anthony et al., 1981; Becker et al., 2013b).

Moreover, bats are the only mammals capable of active flight (Kunz and Pierson, 1994). Enhanced body temperatures and metabolism during flight are similar to febrile conditions in other mammals, which could increase immune function (e.g. antibody production, complement) (O'Shea et al., 2014). This might in part explain why bats can be reservoirs for many emerging viruses. In contrast, bats have low body temperatures during daily torpor and seasonal hibernation (Geiser, 2004). Low body temperatures lead to a decrease in viral replication (Dempster et al., 1966) and to a reduction in host immune function (Bouma et al., 2010; Prendergast et al., 2002). This could lead to a retarded pathogen clearance from bats (George et al., 2011). Additionally, torpor use varies strongly between bat species and within species during the season according to the fluctuating energy expenditure patterns of bats (Otto et al., 2013). Understanding the variation of chiropteran immunity is essential from evolutionary, ecological, conservation, and public health points of views.

In order to evaluate the complex immunity of an individual, it is necessary to use a combination of assays measuring both humoral and cellular effectors of the innate and adaptive immune branches (Adamo, 2004; Matson et al., 2006; Norris and Evans, 2000). We used a combination of species non-specific eco-immunological techniques developed for free-living wildlife (Demas et al., 2011) to assess the immunity of the European bat *Myotis daubentonii*. We measured (i) the bacterial killing activity of plasma against *Escherichia coli* (BKA; functional innate immune defense), (ii) hemagglutination (HA) and (iii) hemolysis (HL) titers, representing the levels of natural antibodies and complement (innate immune defenses), respectively, (iv) the immunoglobulin G (IgG) concentrations (adaptive immune defense), and (v) the total and differential white blood cell (WBC) counts (current state of cellular immunity).

We predicted (i) that female *Myotis daubentonii* would have higher immunity than males similar to most other vertebrate species and (ii) that sexes differ according to their energy demand (e.g. reproductive state). Due to reallocation processes, immune functions should be suppressed during phases of high energy demand, such as pregnancy and lactation in females, and spermatogenesis in males (Encarnação and Dietz, 2006).

## Material and methods

### *Study species*

The Daubenton's bat (Chiroptera, Vespertilionidae: *Myotis daubentonii* Kuhl 1817) is a widespread and abundant Eurasian bat species (IUCN Red List Category: Least Concern). It is distributed from central Norway through Europe to the Mediterranean and northern Asia, with a continuous increase in population size (Stubbe et al., 2008). Females appear from hibernation in early spring in their summer habitat where pregnancy starts immediately after fertilisation. They give birth in late spring (Encarnação, 2012) and lactate during early summer. During pregnancy and lactation, they communally breed in large maternity colonies (Lučan et al., 2009). In contrast, males roost individually or in small aggregations. During late summer, in males spermatogenesis peaks (Encarnação, 2012) and adult females are in the period of post-lactation. Mating occurs mainly in late summer and early autumn before hibernation (Encarnação et al., 2004).

### *Capture of bats*

Daubenton's bats (n = 84) were captured in the years 2013 and 2014 by mist-netting or roost-trapping near Giessen, Hesse, Germany. Sex (males: n = 40, females: n = 44) and reproductive state were visually determined. Females were categorized as not-reproductive, pregnant, lactating or post-lactating. Pregnant bats were distinguished by abdominal palpation. Lactation is indicated by swollen nipples and bald areolae. During post-lactation, females have developed, but flaccid nipples (Racey, 2009). Females in late pregnancy or early lactation were released at the initial capture site without sampling.

Male bats were classified as reproductively active or non-reproductive according to their epididymal filling (non-reproductive: 0% epididymal filling; reproductive: >0% epididymal filling). The degree of distension of the epididymis is useful to assess the reproductive state of bats (Encarnação et al., 2004) because during the reproduction period, it fills with sperm and stores these spermatozoa until mating. This filling can be examined visually since the cauda epididymis is enclosed in a pigmented sheath (Krutzsch, 2000).

Body mass (digital pocket balance CM, Kern & Sohn GmbH, accuracy 0.01 g) and forearm length (callipers, Hydrotec Technologies, accuracy 0.01 mm) were measured. Subadults (total n = 13; males: n = 2, females: n = 11) were identified based on the presence of a black chin-spot in combination with the growth of phalangeal epiphyses (subadults: unfused/translucent) (Anthony, 1988).

### *Blood sampling*

Blood samples were obtained by venipuncture using a lancet to pierce the interfemoral vein. Approximately 50 μL of whole blood was collected using microcapillary tubes coated with heparin-lithium (Microvette® Sarstedt). If blood volumes were sufficient, a blood smear was prepared using a subsample of the whole blood (n = 31). Due to field conditions, the remaining blood was stored on ice (max. 1 h) until centrifugation (2875 × *g*, 10 min) and the plasma was collected. Additionally, 10 μL of whole blood was collected using tubes coated with clotting activator (Microvette® Sarstedt). It was allowed to clot for 1 h before centrifugation (850 × *g*, 10 min). Plasma and serum samples were stored at −20 °C until further analysis. Plasma samples were used to determine BKA (n = 75) and HL/HA titers (each n = 65) while IgG concentration (n = 54) was measured in serum samples.

### *Bacterial killing activity*

A bacterial-killing assay was conducted after French and Neuman-Lee (2012) with modifications as described by Strobel et al. (2015). The assay measures the killing activity of plasma against *Escherichia coli*, a gram-negative bacteria, which was used because it is ubiquitous and thus guarantees that bats show effec-

tive constitutive innate immune responses which are independent of the individual pathogen-exposure history (Millet et al., 2007). Moreover, i) this microbe is the most commonly used bacteria in ecoimmunological studies thus provides possibilities for comparison with other species (French and Neuman-Lee, 2012; Heinrich et al., 2016), ii) its killing is complement dependent (Moore et al., 2011) and iii) the method has been previously used in several bat species (Allen et al., 2009; Schneeberger et al., 2013b; Becker et al., 2017, 2018). Briefly, *E. coli* (ATCC #8739, Epower, Doenitz Pro-Lab) were reconstituted in Phosphate Buffered Solution (PBS) and diluted to a working concentration of $10^5$ bacteria/mL. In 96-well round bottom microplates (Roth) 2 μL of plasma was diluted 1:8 in PBS. To each well, 6 μL of bacteria working solution was added and incubated at 37 °C. Tryptic Soy Broth (Sigma-Aldrich) was added and absorbance was read at 300 nm (Infinite M200, Tecan). Plates were incubated at 37 °C for 12 h, shook for 60 s to dissolve precipitation, and the absorbance was measured again. All plates were run with positive (without plasma) and negative controls (without bacteria). To determine BKA, the percent of killed bacteria relative to positive control was calculated.

*Hemolysis/hemagglutination titers*

The hemolysis-hemagglutination assay was conducted as described by Matson et al. (2005) with minor modifications for low sample volumes (Sparkman and Palacios, 2009) and mammals (Heinrich et al., 2017). Beginning with a 1:2 dilution in PBS in the first column of a microtiter plate, serial twofold dilutions of 10 μL plasma samples were made through to column 11. The twelfth row contained only PBS as negative control. To each well, 10 μL of a 1% chicken red blood cell suspension (Fiebig Nährstofftechnik) was added. Plates were incubated at 37 °C and photographed after 110 min for agglutination (natural antibodies) and after additional 70 min for lysis (complement). For each sample, images were randomized and scored blindly.

*Immunoglobulin G concentration*

Immunoglobulin G concentration was measured using a Protein G ELISA assay developed by Schneeberger et al. (2014). High-binding 96-well plates (immunoGrade™, Roth) were coated with 100 μL diluted serum samples and positive controls (pooled and aliquoted sera of *M. daubentonii*) (1:10,000 in 50 mM $NaHCO_3$, pH 9.5) in triplicate and incubated (1 h, 37 °C). Plates were washed in Tris-buffered saline/Tween-20 (TBS-T, Roth) and blocked with 200 μL of 1% gelatine (Merck) solution and incubated (30 min, 37 °C). After washing, 100 μL of Protein G-horseradish peroxidase conjugate solution (Merck Millipore, 1:12,000 in TBS-T, pH 7.4) was added and incubated (30 min, RT). Wells were washed and incubated with 100 μL TMB [10% 3,3', 5,5'-tetramethylbenzidine (SouthernBiotech) in DMSO (Sigma-Aldrich) diluted 1:100 in phosphate-citric buffer pH 5.0 and mixed with 30% $H_2O_2$ (Roth)]. The reaction was stopped after 15 min with 100 μL 1 M sulphuric acid (Roth). Absorbance was read immediately at 450 nm (reference wavelength, 620 nm) (Infinite M200, Tecan). Negative controls were run on each plate.

*White blood cell counts*

Blood smears were stained with May-Gruenwald's (Roth) and Giemsa (Roth) solutions. For each individual, total and differential WBC counts were performed. For total WBC counts, the mean of cell counts in five fields at 100× magnification were calculated (Schneeberger et al., 2013a, 2014). Differential WBC counts were estimated by counting leukocyte types at 1000× magnification under oil immersion until a total of 100 leukocytes was reached in non-overlapping random fields (Fokidis et al., 2008). Relative numbers (%) of eosinophils, basophils, neutrophils, lymphocytes, and monocytes were calculated (Schneeberger et al., 2013b). Absolute numbers were estimated by multiplication of relative numbers with total WBC counts. Slides were observed by a single observer blinded in respect to individual data. Stress can alter immunity (Apanius, 1998), therefore, the neutrophil-to-lymphocyte ratio was calculated as an indicator for stress (Becker et al., 2017; Seltmann et al., 2017).

**Table 1**
Descriptive statistics (mean ± standard deviation and sample size in brackets) for immune response defined as bacterial killing activity (BKA), hemagglutination (HA titer), hemolysis (HL titer) and immunoglobulin G (IgG OD) in females and male *Myotis daubentonii* of different reproductive status.

| | BKA (%) | HA titer | HL titer | IgG (OD) |
|---|---|---|---|---|
| females | | | | |
| total | 85.7 ± 7.5 (n = 40) | 8.4 ± 1.2 (n = 35) | 3.5 ± 1.3 (n = 35) | 0.121 ± 0.018 (n = 36) |
| not reproductive | 89.0 ± 6.3 (n = 15) | 8.6 ± 0.9 (n = 10) | 3.3 ± 0.9 (n = 10) | 0.117 ± 0.021 (n = 15) |
| pregnant | 83.5 ± 8.9 (n = 7) | 7.6 ± 1.3 (n = 9) | 3.4 ± 1.0 (n = 9) | 0.140 ± 0.010 (n = 6) |
| lactating | 80.1 ± 9.6 (n = 6) | 8.5 ± 1.8 (n = 6) | 5.1 ± 0.9 (n = 6) | 0.119 ± 0.012 (n = 6) |
| post-lactating | 85.2 ± 6.0 (n = 12) | 8.8 ± 1.0 (n = 10) | 2.7 ± 1.2 (n = 10) | 0.115 ± 0.014 (n = 9) |
| males | | | | |
| total | 91.4 ± 9.1 (n = 35) | 8.3 ± 1.5 (n = 30) | 2.5 ± 1.6 (n = 30) | 0.120 ± 0.018 (n = 18) |
| not reproductive | 93.5 ± 7.2 (n = 17) | 8.1 ± 1.6 (n = 18) | 2.4 ± 1.9 (n = 18) | 0.122 ± 0.017 (n = 10) |
| reproductive active | 89.4 ± 10.4 (n = 18) | 8.6 ± 1.2 (n = 12) | 2.7 ± 1.1 (n = 12) | 0.118 ± 0.020 (n = 8) |

*Data analysis*

All statistical analyses were carried out using STATISTICA software package (V12, StatSoft Inc.). Adults and subadults did not differ significantly from each other and were therefore pooled for further analysis. Before performing statistical comparisons, Shapiro–Wilk tests were used to test variables for normality. Sex differences in plasma immune parameters were examined by comparing non-reproductive males and females with a *t*-test for independent samples or Mann–Whitney *U* (MWU)-test when normality was not met. To test for the effect of reproductive states on variation of each of the plasma immune measures, males (*t*-test, MWU-test; categories: not reproductive, reproductive active) and females (analysis of variance (ANOVA); categories: not-reproductive, pregnant, lactating, post-lactating) were analysed separately. Cell measurements were only obtained for not reproductive/reproductively active males and post-lactating/lactating females. Therefore, the influence of the reproductive state on the cell measurements was analysed using a nested general linear model (GLM) with the explanatory factors "sex" and "reproductive state". All pair wise comparisons of mean differences were conducted using Tukey's HSD post hoc comparisons.

## Results

For the humoral immune parameters of male and female *M. daubentonii* (Table 1), BKA was higher in males compared to females (MWU-test, U = 50.0, $P = 0.006$; Fig. 1). In contrast, IgG concentration (*t*-test, t = 1.14, $P = 0.270$), HL titer (*t*-test, t = −0.91, $P = 0.373$), and HA titer (*t*-test, t = −1.66, $P = 0.108$) did not differ between sexes.

For male bats, reproductive states did not influence humoral immune parameters (BKA, MWU-test, U = 121.0, $P = 0.299$; HA titer: MWU-test, U = 89.0, $P = 0.434$; IgG concentration, *t*-test, t = −0.55,

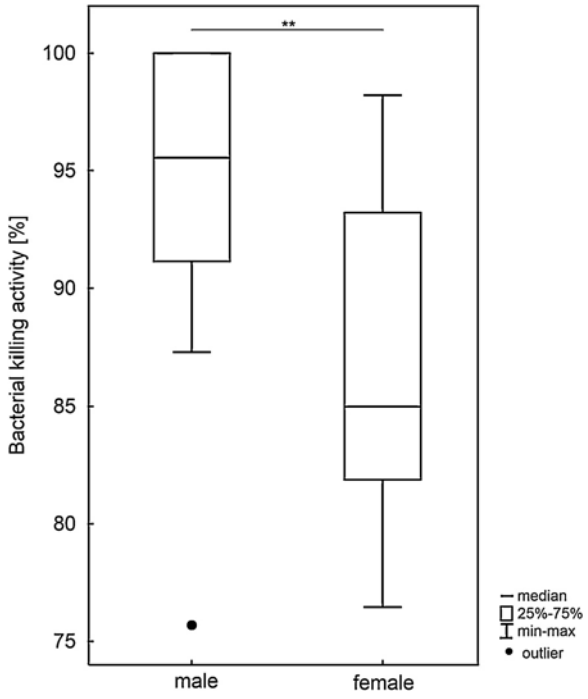


**Fig. 1.** Median bacterial killing activity (%) of non-reproductive male and female *Myotis daubentonii*. Significant differences are indicated by asterisks (MWU-test, $P < 0.01$). Boxes indicate 25–75% percentiles, whiskers minimum and maximum values, and dots demonstrate outliers.

$P = 0.591$; HL titer: *t*-test, t = 0.52, $P = 0.610$). Females differed significantly in IgG concentration and HL titer in relation to their reproductive state. For IgG concentration (ANOVA, F = 3.2, $P = 0.035$), pregnant females had significantly higher values than non-reproductive (Tukey's HSD, $P = 0.036$; Fig. 2) and individuals during post-lactation (Tukey's HSD, $P = 0.042$; Fig. 2). Hemolysis titer (ANOVA, F = 6.9, $P = 0.001$) was higher in lactating compared to non-reproductive (Tukey's HSD, $P = 0.012$), pregnant (Tukey's HSD, $P = 0.021$), and female bats during post-lactation (Tukey's HSD, $P = 0.0006$) (Fig. 2). Bacterial killing activity (ANOVA, F = 2.2, $P = 0.111$) and HA titer (ANOVA, F = 1.7, $P = 0.190$) did not differ between reproductive states for females.

In case of the immune cells (Table 2), the nested GLM ($R^2 = 0.36$, d.f. = 3, $P = 0.006$) revealed significant influence of the reproductive state on total WBC counts (GLM, $P = 0.004$) whereas there was no difference between sexes (GLM, $P = 0.248$). Non-reproductive males had more total WBCs than reproductive males (Tukey's HSD, $P = 0.047$). No difference between pregnant and lactating females could be observed (Tukey's HSD, $P = 0.079$). The nested GLM ($R^2 = 0.78$, d.f. = 3, $P < 0.0001$) demonstrated that the number of neutrophils was influenced by the factors sex (GLM, $P < 0.0001$) and reproductive state (GLM, $P < 0.0001$). Female bats had significantly more neutrophils than males (Tukey's HSD, $P = 0.0001$). Comparing the respective reproductive states, pregnant females had higher numbers of neutrophils than lactating individuals (Tukey's HSD, $P = 0.0002$). In contrast, for males, no effect of the reproductive state on neutrophil counts was observed (Tukey's HSD, $P = 0.691$). The model (GLM, $R^2 = 0.25$, d.f. = 3, $P = 0.048$) showed that lymphocyte counts were influenced by the reproductive state (GLM, $P = 0.040$), but not by the sex (GLM, $P = 0.164$) of the individuals. However, post-hoc comparison did not show any significant difference between the reproductive states (Tukey's HSD, females: lactating/pregnant, $P = 0.961$; males: non-reproductive/reproductive, $P = 0.060$). The nested GLM revealed differences between the neutrophil-to-lymphocyte ratios (GLM, $R^2 = 0.70$, d.f. = 3, $P < 0.0001$). It was significantly influenced by sex (GLM, $P < 0.0001$) and reproductive state (GLM, $P = 0.0001$). In general, females had higher neutrophil-to-lymphocyte ratios than males (Tukey's HSD, $P = 0.0001$). The neutrophil-to-lymphocyte ratio between reproductive states differed within females. Preg-

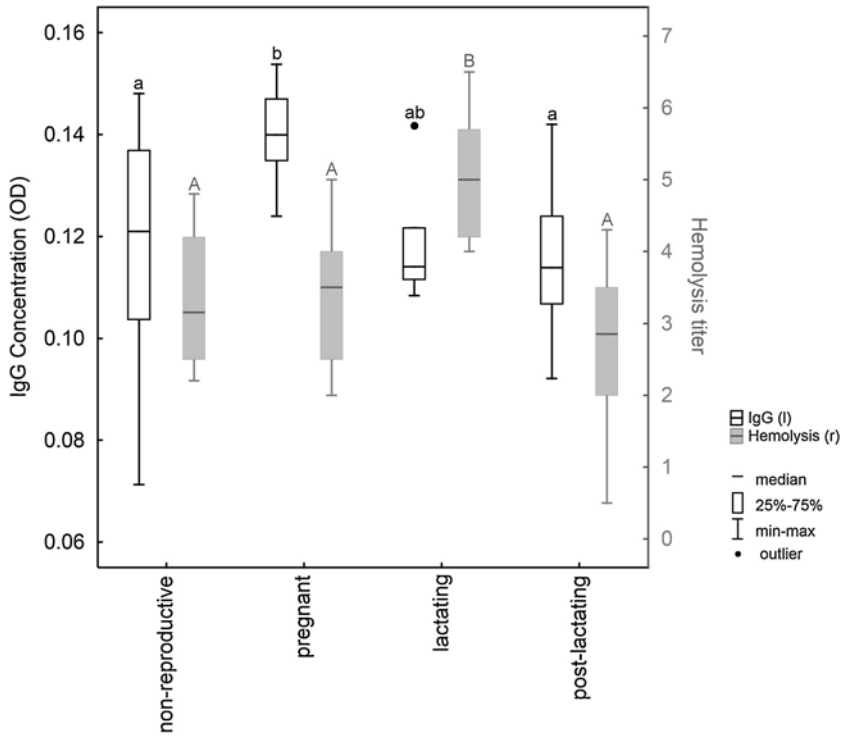


**Fig. 2.** Variability in mean IgG concentration (OD, left y-axis, white) and hemolysis titer (right y-axis; grey) between non-reproductive, pregnant, lactating, and post-lactating female *Myotis daubentonii*, respectively. Boxes with different letters are significantly different (small letters: IgG concentration, capital letters: hemolysis; *t*-tests, $P < 0.05$). Boxes indicate 25–75% percentiles, whiskers minimum and maximum values, and dots demonstrate outliers.

**Table 2**
Descriptive statistics (mean ± standard deviation) for hematological parameters in female and male *Myotis daubentonii* of different reproductive status. Data are given as percentage and absolute numbers per visual leukocyte subtypes. The N:L represents the neutrophil-lymphocyte ratio whereas WBCs the white blood cells per field.

| | n | WBCs per field | Eosinophils | | Basophils | | Neutrophils | | Lymphocytes | | Monocytes | | N:L ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % | per field | % | per field | % | per field | % | per field | % | per field | |
| females | | | | | | | | | | | | | |
| total | 11 | 16.8 ± 9.8 | 4.5 ± 6.8 | 0.5 ± 0.6 | 2.7 ± 2.5 | 0.3 ± 0.4 | 45.3 ± 20.7 | 8.0 ± 5.9 | 42.7 ± 18.2 | 7.3 ± 6.6 | 4.8 ± 3.5 | 0.6 ± 0.4 | 1.48 ± 1.26 |
| pregnant | 5 | 22.9 ± 7.1 | 1.8 ± 1.5 | 0.4 ± 0.3 | 0.6 ± 0.9 | 0.0 ± 0.2 | 62.4 ± 16.5 | 12.6 ± 3.2 | 32.6 ± 17.0 | 5.2 ± 7.2 | 2.6 ± 1.7 | 0.7 ± 0.5 | 2.42 ± 1.32 |
| lactating | 6 | 11.8 ± 9.2 | 6.7 ± 8.7 | 0.6 ± 0.8 | 4.5 ± 1.9 | 0.3 ± 0.4 | 31.0 ± 9.8 | 2.8 ± 2.3 | 51.2 ± 15.6 | 4.0 ± 6.1 | 6.7 ± 3.6 | 0.6 ± 0.2 | 0.70 ± 0.37 |
| males | | | | | | | | | | | | | |
| total | 20 | 13.6 ± 8.0 | 2.2 ± 3.0 | 0.4 ± 0.6 | 3.7 ± 2.4 | 0.4 ± 0.2 | 14.7 ± 10.0 | 2.2 ± 2.1 | 75.5 ± 11.6 | 10.2 ± 6.4 | 4.0 ± 3.4 | 0.5 ± 0.5 | 0.22 ± 0.19 |
| not reproductive | 9 | 18.6 ± 8.3 | 3.0 ± 3.0 | 0.6 ± 0.8 | 2.7 ± 1.5 | 0.4 ± 0.2 | 14.4 ± 8.0 | 1.6 ± 2.1 | 76.1 ± 12.0 | 12.8 ± 6.7 | 3.8 ± 3.1 | 0.4 ± 0.5 | 0.21 ± 0.15 |
| reproductive active | 11 | 9.6 ± 5.0 | 1.5 ± 2.9 | 0.0 ± 0.3 | 4.5 ± 2.8 | 0.4 ± 0.2 | 14.9 ± 11.8 | 0.8 ± 1.9 | 74.9 ± 11.9 | 6.0 ± 3.5 | 4.2 ± 3.7 | 0.3 ± 0.4 | 0.23 ± 0.23 |

nant females had higher ratios than lactating individuals (Tukey's HSD, $P = 0.0003$). For males, no influence could be observed (Tukey's HSD, $P = 1.000$). There was no effect of sex or reproductive state on the counts of basophils (GLM, $P = 0.05$), monocytes (GLM, $P = 0.56$) and eosinophils (GLMs, $P = 0.21$).

## Discussion

We predicted that female bats have higher immunity than males similar to other vertebrates (Becker et al., 2017; Schuurs and Verheul, 1990). Our results did not confirm this hypothesis, as only BKA and neutrophil numbers differed between sexes. Male individuals had a higher BKA, but less neutrophils compared to females. Similar observations were made for humans and it is possible that the level of oestrogen influences these cell counts (Bain and England, 1975; Hsieh et al., 2007).

We further predicted to find within-sex differences in accordance to the reproductive state of individuals. During active reproductive periods, such as pregnancy or lactation, immune functions should be suppressed as the energy availability for immune functions is limited (French et al., 2007). This hypothesis was partly confirmed, as the modulation of immune parameters during the reproductive states varied differently in the two sexes. For male bats, no effect of the reproductive state on humoral immune parameters could be observed. However, reproductive active males had less total WBCs compared to non-reproductive individuals. Reduced WBC numbers in reproductive males were already described for other mammals, for example ground squirrels (*Spermophilus parryii plesius*) (Boonstra et al., 2001). This might indicate a trade-off between immunity and reproduction, which is in part explained by the effects of different hormones on the immune system. A lower cellular immunity could be mediated by testosterone. In European starlings (*Sturnus vulgaris*), for example, this hormone has a direct suppressive effect on cellular immune responses (Duffy et al., 2000). During reproduction, testosterone is elevated in male bats (Martin and Bernhard, 2000), which can be associated with a decrease in WBCs (Al-Afaleq and Homeida, 1998). The effect of testosterone on the components of the immune system is controversial (Buchanan et al., 2003; Greiner et al., 2010; Roberts et al., 2004). Moreover, the mechanism of immune regulation is complex and it is possible that other substances, such as stress hormones, might also interact with the immune system (Folstad and Karter, 1992). However, the stress indicator, neutrophil-to-lymphocyte ratio, was not increased in reproductive males. As reproduction is a period of high energy demands (Gittleman and Thompson, 1988), the decrease in WBCs could be a consequence of the energetic trade-off between reproduction and immunity in male *M. daubentonii* in favor of reproductive success.

For female bats there was a clear influence of the reproductive states on both humoral and cellular immune parameters. Pregnant females had more neutrophils and higher IgG concentrations compared to females of other states. During lactation, HL titer was increased. These differences could be explained by specific effects of sex hormones (Da Silva, 1999), physiological trade-offs mediated by energy demands (Martin et al., 2008), varying pathogen exposure (Schneeberger et al., 2013b), or by synergistic effects of these factors. Sex hormone levels are elevated during pregnancy (Martin and Bernhard, 2000) and can induce the release of neutrophils from the bone marrow, leading to an increased cell number (reviewed in Bouman et al., 2005), especially in the early stages of pregnancy (Valdimarsson et al., 1983). High neutrophil counts of *M. daubentonii* led to an enhanced neutrophil-to-lymphocyte ratio in pregnant compared to lactating females. The increased IgG concentration during pregnancy could be explained by a higher infection rate in female bats during maternity period (Mühldorfer et al., 2011; Plowright et al., 2008) and/or by the need to pro-

vide passive immunity for their offspring. Female individuals of *M. daubentonii* form large maternity groups (Lučan et al., 2009), which enhances the risk of pathogen and parasite spread (Drexler et al., 2011; Hayman et al., 2013). Such high antibody concentrations could be a relatively cheap response to the high pathogen exposure because it is energetically less expensive than the development of cell-mediated or induced innate immunity (Martin et al., 2008). For example, for the greater mouse-eared bat (*Myotis myotis*) it is known, that the T-cell response is reduced in pregnant females, probably due to a sex-hormones-modulated physiological trade-off during pregnancy (Christe et al., 2000). Female mammals transfer IgGs to their offspring (Borghesi et al., 2014). The mechanism in bats is not clear, but it is speculated for pteropid bats, that they use a combination of transfer via placenta and colostrum (Epstein et al., 2013). As the amount and diversity of mothers' blood and transferred antibodies correlate (Grindstaff, 2008; Lemke et al., 2003), the high concentration of IgG during pregnancy might indicate an antibody transfer via placenta in *M. daubentonii*.

During lactation, HL titer significantly increased. Maternity roosts are inhabited by high densities of lactating females and their young (Dietz et al., 2009). Both are immunologically compromised, providing an ideal environment for transmission of pathogens (Gloza-Rausch et al., 2008; Mühldorfer et al., 2011). Complement proteins are constitutive components of the innate immune system and act as the first-line defense again pathogens (including viruses) (Ochsenbein et al., 1999; Zipfel, 2009). The costs of maintenance and use of this innate defense are thought to be low (Lee, 2006). As lactation is the most energy-demanding period in the life of female mammals (Hammond and Diamond, 1997; Thompson, 1993), it seems that lactating *M. daubentonii* rely on a high immune responsiveness of this immune component.

Effects of sex and reproductive state on immune parameters of *M. daubentonii* were observed. These variations differed between sexes possibly due to different modulations of immune components. Changes in immune responses in bats might be explained by the combined influences of hormones, energetics, and social behaviour. These results highlight the need for more studies investigating intraspecific immunological differences in combination of hormonal and energetic measurements for a better understanding on how bats manage physiological trade-offs. We furthermore emphasise, that it is not only important to measure humoral and cellular components of both branches of the immune system of wild mammals, but it is also essential to consider the influence of sex and physiological status when interpreting the observed values (Pap et al., 2010).

## Ethical approval

Experiments were ethically approved and permitted by the nature conservation authority according to BNatSchG §45 (7) (No. 53.2 – V/53.2-R 22-3 (3)) and by the animal care authority of the administrative district of Giessen, federal state of Hesse (Germany) according to TierSchG §8 (1) (No. V54-19 c 20 15h 01 GI 15/8Nr. 15/2013).

## Conflict of interest

The authors declare that they have no conflict of interest.

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