

Handling Stress and Sample Storage Are Associated with Weaker Complement-Mediated Bactericidal Ability in Birds but Not Bats*

Daniel J. Becker^{1,2,†}

Gábor Á. Cziráj³

Agnieszka Rynda-Apple¹

Raina K. Plowright¹

¹Department of Microbiology and Immunology, Montana State University, Bozeman, Montana 59717; ²Center for the Ecology of Infectious Diseases, University of Georgia, Athens, Georgia 30602; ³Department of Wildlife Diseases, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

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ABSTRACT

Variation in immune defense influences infectious disease dynamics within and among species. Understanding how variation in immunity drives pathogen transmission among species is especially important for animals that are reservoir hosts for zoonotic pathogens. Bats, in particular, have a propensity to host serious viral zoonoses without developing clinical disease themselves. The immunological adaptations that allow bats to host viruses without disease may be related to their adaptations for flight (e.g., in metabolism and mediation of oxidative stress). A number of analyses report greater richness of zoonotic pathogens in bats than in other taxa, such as birds (i.e., mostly volant vertebrates) and rodents (i.e., nonvolant small mammals), but immunological comparisons between bats and these other taxa are rare. To examine interspecific differences in bacterial killing ability (BKA), a functional measure of overall constitutive innate immunity, we use a phylogenetic meta-analysis to compare how BKA responds to the acute stress of capture and to storage time of frozen samples across the orders Aves and Chiroptera. After adjusting for host phylogeny, sample size, and total microbe colony-forming units, we find preliminary evidence that the constitutive innate immune defense of bats may be more resilient to handling stress and storage time than that of birds. This pattern was also similar when we analyzed the proportion of nonnegative and positive effect sizes per species,

using phylogenetic comparative methods. We discuss potential physiological and evolutionary mechanisms by which complement proteins may differ between species orders and suggest future avenues for comparative field studies of immunity between sympatric bats, birds, and rodents in particular.

Keywords: acute stress, bacterial killing assay, bacterial killing ability, ecoimmunology, *Escherichia coli* ATCC 8739, innate immunity, wildlife.

Introduction

Immunology is central to determining the outcome of host-pathogen interactions (Hawley and Altizer 2011). Individual variation in immune defense influences within-host processes such as resistance and tolerance to infection and the clearance of infectious agents (Jolles et al. 2015). Variability in immune defense across species may also drive variability in the propensity to transmit pathogens within and between host species and to host zoonotic pathogens (Paull et al. 2011; VanderWaal and Ezenwa 2016). For example, trade-offs between innate and adaptive immunity driven by host life history likely drive reservoir host competence in several systems. Fast-lived amphibians likely invest less in immune defenses and show lower tolerance to *Ribeiroia* infections (Johnson et al. 2012), and fast-lived mammals show weaker adaptive immunity that may explain competence for *Borrelia burgdorferi* (Previtali et al. 2012). More broadly, immunological differences between host taxa could help explain large-scale variation in reservoir status, pathogen richness, and spillover risk (Bean et al. 2013; Stephens et al. 2016).

Bats (order: Chiroptera) have been increasingly identified as reservoir hosts for zoonotic pathogens (Mühldorfer 2013; Plowright et al. 2015), including Hendra and Nipah virus, SARS (severe acute respiratory syndrome) coronavirus, *Bartonella*, and Marburg virus, among others (Li et al. 2005; Halpin et al. 2011; Amman et al. 2014; Veikkolainen et al. 2014; Becker et al. 2018a). At the species level, bats host more zoonotic viruses than other mammal orders (Olival et al. 2017). However, this distinction cannot be fully explained through bat life history alone (e.g., their exceptional longevity, given small body size; Foley et al. 2018) or their high degree of sympatry (Luis et al. 2013).

Immunological differences between bats and other taxa could drive observed differences in viral richness (Schountz et al. 2017). Bats exhibit few clinical symptoms when infected with several viruses that are virulent in other taxa (Williamson et al. 2000; Middleton et al. 2007), can fail to develop fever and leukopenia

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†Corresponding author; email: daniel.becker3@montana.edu.

when challenged with lipopolysaccharides (Stockmaier et al. 2015; but see Schneeberger et al. 2013a and Weise et al. 2017), can have complement proteins that are insensitive to temperature changes (Hatten et al. 1973), and can constitutively express interferon alpha (Zhou et al. 2016). However, some aspects of their immune systems are similar to those of other species. For example, immune cells and immunoglobulins are akin to those in humans and rodent models (Baker et al. 2013). As bats are the only volant mammals, evolution of flight has been proposed as a mechanism for viral control (Zhang et al. 2013; O'Shea et al. 2014). Bats increase their metabolic rate during flight, more so than running rodents and flying birds (Speakman et al. 2003; Bundle et al. 2007), and high metabolism can enable a stronger immune response (Książek and Konarzewski 2012). The costs of mounting an immune response could thus be less expensive for bats (O'Shea et al. 2014); however, support for this idea is equivocal (Cabrera-Martínez et al. 2018; Guerrero-Chacón et al. 2018). Yet because flight also elevates body temperature to mirror a febrile response, daily activation of the immune system with flight could facilitate tolerating viral infections (O'Shea et al. 2014). The evolution of flight was also likely followed by adaptations to mediate the large oxidative-stress burdens incurred during this metabolically costly activity, which could further enable viral tolerance by physiological mechanisms such as dampened interferon responses (Zhang et al. 2013; Xie et al. 2018).

Such hypotheses for flight as a driver of immunological distinction and viral tolerance could be explicitly tested with comparative analyses. Immunological comparisons between bats, birds (i.e., similarly sized volant, and some nonvolant, vertebrates), and rodents (i.e., nonvolant, similarly sized mammals) would be especially insightful, given that immunological investment varies with species body mass (Schneeberger et al. 2013b; Tian et al. 2015), that all three endothermic orders are reservoir hosts for various zoonoses (Reed et al. 2003; Han et al. 2016), that bats and birds both display exceptional longevity (Munshi-South and Wilkinson 2010), and that ancestral members of these orders are likely to have had characteristics of animals that survived the K-T extinction (Wang et al. 2011). We present one such preliminary test by comparing bactericidal activity across the orders Aves and Chiroptera. While we aimed to assess immunological comparison between Aves, Chiroptera, and Rodentia, we excluded the latter order because of a lack of relevant studies. Although quantifying immune defense across species can be challenging (Demas et al. 2011), functional tests such as the bacterial killing assay can overcome some limitations by measuring the capacity of blood, plasma, or serum to remove pathogens or limit their replication *ex vivo* with small sample volumes and without reliance on species-specific reagents (Millet et al. 2007; Liebl and Martin 2009; French and Neuman-Lee 2012). Depending on the bacterium and sample type, bacterial killing ability (BKA) is mediated by phagocytes (e.g., macrophages, neutrophils), opsonizing proteins (e.g., complement and acute-phase proteins), and natural antibodies (i.e., immunoglobulins M and A) and can be considered a functional measure of overall constitutive innate immune defense (Tielemans et al. 2005).

Previous studies have shown that BKA varies systematically across species and with traits such as pace of life and roost permanence within the Aves, Rodentia, and Chiroptera (Tielemans et al. 2005; Martin et al. 2007; Previtali et al. 2012; Schneeberger et al. 2013b). However, such measures of immune defense have not been examined across these orders, primarily as the assay must be optimized on a per-species basis to identify a sample dilution that yields average killing of approximately 50% (French and Neuman-Lee 2012). Direct comparison of the BKA between species therefore relies on using the same dilution factor across species (e.g., Matson et al. 2006; Martin et al. 2007) or serially diluting all samples in a study (Heinrich et al. 2016). Alternatively, comparison of the dilution killing curve across species can also facilitate cross-species analyses when such data are available (Heinrich et al. 2016). Difference in environmental context across studies also represents another barrier to directly comparing BKA when species are not sampled in sympatry, as assay optimization must also consider such sources of immunological variation (French and Neuman-Lee 2012). Given these limitations, cross-species comparisons could instead be facilitated by systematically investigating differences in how BKA responds to variable study conditions across taxa. Several studies have suggested that BKA of bats and birds may respond to acute stress and temperature change differently, which would facilitate cross-species comparisons of an effect size. For example, an hour of acute stress (i.e., capture) reduced BKA by up to 40% in volant Neotropical birds (Matson et al. 2006), reflecting the immunosuppressive effects of stress (Padgett and Glaser 2003). However, an acute stressor of longer duration did not affect BKA in the common noctule bat (*Nyctalus noctula*; Strobel et al. 2015) and the Brazilian free-tailed bat (*Tadarida brasiliensis*; Allen et al. 2008), suggesting that stress hormones do not affect components of innate immunity in bats. Similarly, while sample storage impaired BKA in house sparrows (*Passer domesticus*; Liebl and Martin 2009), freezing had little impact on BKA in Neotropical bats (Schneeberger et al. 2013b; Becker et al. 2017), suggesting potential differences in the temperature resilience of immune components in plasma between taxa (Jacobs and Fair 2016). We use a phylogenetic meta-analysis to test the generality of these interorder relationships while accounting for host phylogeny, sample size, and method variations. Our results suggest preliminary immunological differences between bats and birds that can be more robustly tested in field studies with more comprehensive immune measures.

Methods

Effect Size Collection

As we aimed to obtain data on the relationship between handling or storage time and BKA across bats, birds, and rodents, we performed systematic searches in Google Scholar and Web of Science, using the following string: ("bacterial killing ability" OR "bacterial killing assay" OR "bacterial killing capacity") AND (bat* OR bird* OR rodent*). As inclusion criteria, we first included only studies that quantified BKA in a wild bat, bird, or rodent (fig. S1; figs. S1–S4 are available online; Moher et al.

2009). After screening papers by title and abstract for assessment of BKA in these taxa, we excluded studies if test statistics were not reported or if $n < 4$ subjects were examined ($n = 8$ studies). To increase our sample, we included studies cited in the systematically identified papers ($n = 3$ studies). We limited our analyses to assays with *Escherichia coli* ATCC 8739, as no other microbe was used for bats in our data set. As BKA is mediated by different effectors (cellular or humoral), depending on the pathogen and sample type, we focused on studies that tested complement-mediated BKA and recorded whether assays used whole blood or plasma and serum (Millet et al. 2007; Moore et al. 2011). Only one record used the nanodrop spectrometry method (Liebl and Martin 2009), which can produce results less variable than those of microplate-based spectrometry (French and Neuman-Lee 2012); we therefore excluded this record from analyses.

From each test of relationships between BKA and handling or storage time, we recorded the host species, assay type (i.e., agar plate or microplate spectrometry), sample volume, microbe volume and concentration, sample type, covariate (i.e., handling or storage time), maximum minutes animals were held before sampling, maximum days samples were frozen before assay, freezing temperature, and whether conditions were imposed experimentally (e.g., whether authors manipulated handling or storage time vs. an analysis of observed variation). We multiplied microbe volume and concentration to generate total colony-forming units (CFUs). We also recorded sample size, test statistics, and effect direction (i.e., positive or negative association between handling or storage time and BKA). Only one record did not report effect directionality (Jacobs and Fair 2016); we therefore performed our analyses with both negative and positive values assigned (Bentz et al. 2016), although we present only results from the former condition.

We reported effect size as the correlation-based r between handling or storage time and BKA. As r was rarely directly reported, we converted other test statistics (e.g., χ^2 , d , F , t) into correlation coefficients (Wolf 1986; Borenstein et al. 2009). If test statistics were not reported, we derived Cohen's d for the difference between two means (e.g., before and after handling stress). When authors reported only no significant relationship between these handling or storage times and BKA, we assigned an r of 0 (Rosenthal and DiMatteo 2001). We assigned negative values to r when BKA was lower for animals held longer between capture and sampling and for samples stored longer before assay. We used the R package *metafor* to convert directional r effect sizes into Fisher's Z (Zr) as a normalizing transformation (Viechtbauer 2010; R Development Core Team 2013).

Phylogenetic Meta-analysis

To test whether effect sizes differ between bats and other similarly sized taxa (i.e., birds), we fitted hierarchical mixed-effects meta-analysis models (MEMs) with observation, study, and species set as random effects (Konstantopoulos 2011). We controlled for phylogenetic nonindependence by specifying the covariance structure of the species random effect by using a

phylogenetic correlation matrix derived from the Open Tree of Life (Hinchliff et al. 2015). We used the *rotl* and *ape* packages to extract the phylogeny, prune the tree to our species, resolve multichotomies, and provide branch lengths using Grafen's method (Grafen 1989; Paradis et al. 2004; Michonneau et al. 2016). We nested observations within study to account for unit-level variance and study pseudoreplication, as half our studies contained more than one effect size (5/10).

We compared a candidate set of MEMs to identify the predictors of correlations between handling or storage time and BKA of the species order. We restricted the number of covariates per model ($k = 1-3$), given our limited sample size of 27 records (Burnham and Anderson 2002). We considered an intercept-only model (i.e., a random-effects meta-analysis), along with univariate and additive models with species order, assay type, sample type, sample volume, total CFUs, and BKA covariate assessed (i.e., handling or storage time); we also considered an interaction between the latter and species order. We fitted all models to a reduced data set free of missing values ($n = 25$; two studies did not report microbe concentration). We used maximum likelihood (ML) to compare models with the Akaike information criterion corrected for small sample size (AICc; Burnham and Anderson 2002). We refitted models with restricted ML (REML) to obtain unbiased estimates of variance components, from which we calculated an R^2 as the proportional reduction in summed variance components per MEM, compared with summed variance components of a model without predictors (López-López et al. 2014). We calculated Akaike weights (w_i) to facilitate model comparison, considered MEMs within two $\Delta AICc$ to be competitive, and visualized the top MEMs by back-transforming Zr into r for interpretability.

We next stratified data by BKA covariate (handling time: $n = 19$; storage time: $n = 8$), refitted MEMs with ML and order as a predictor, and used likelihood ratio tests to assess whether inclusion of confounding variables (i.e., maximum minutes animals were held before sampling, maximum days samples were frozen before assay, freezing temperature) modified relationships with order.

Phylogenetic Comparative Methods

We complemented our phylogenetic meta-analysis with a comparative study on species-level data by using weighted phylogenetic generalized least squares (WPGLS; Garamszegi 2014). We used the *nlme* package and WPGLS models fitted with ML to quantify Pagel's λ in the logit-transformed proportions of (1) non-negative and (2) positive effect sizes while accounting for species sample size (Pagel 1999; Pinheiro and Bates 2010). We also fitted WPGLS models with order as a covariate to test whether both these logit proportions varied between species orders.

Publication Bias

Finally, we assessed evidence of publication bias, the preferential publication of significant over nonsignificant results or those with a small effect size (Rosenthal 1979). We first used funnel plots of effect sizes against standard errors to visualize

potential bias for data stratified by each species order; low bias is expected when effects with high precision remain close to the mean and effects with low precision are spread symmetrically from the mean (Egger et al. 1997). We next tested for funnel plot asymmetry with rank correlation tests (Sterne and Egger 2005). Finally, we used the trim-and-fill method to estimate the number of records potentially missing because of publication bias and whether this estimate differed from 0 (Duval and Tweedie 2000).

We note that all these analyses involve small sample sizes because of our inclusion criteria (i.e., relationship between BKA in bats, birds, or rodents and acute stress or storage time), which can increase the risk of conducting a Type II error. While our models include weighting schemes to provide more precise coefficient estimates and increase power in the face of small sample size (Cohn and Becker 2003), our results should still be interpreted as preliminary and are presented in the spirit of encouraging future empirical tests in immunological differences between taxa.

Results

Data Set Description

We identified 10 studies assessing the relationship between handling or storage time and BKA, resulting in 27 unique records encompassing the orders Aves ($n = 18$) and Chiroptera ($n = 9$). While our systematic search identified studies of BKA in Rodentia ($n = 9$ studies, compared to $n = 8$ in Chiroptera and $n = 16$ in Aves), no rodent studies reported statistical tests related to effects of handling or storage time (fig. S1); therefore, our analyses were able to compare only effect sizes between Aves and Chiroptera. A PGLS model using data from EltonTraits showed that body mass did not vary between these bat and bird species ($F_{1,11} = 0.64$, $P = 0.44$; Wilman et al. 2014). All data and the phylogeny are available in the Dryad Digital Repository: <https://dx.doi.org/10.5061/dryad.5618c1m> (Becker et al. 2019).

Records were divided evenly between BKAs based on agar plate ($n = 16$) and microplate spectrometry methods ($n = 11$), whereas assays predominantly used plasma ($n = 21$) rather than blood ($n = 6$). Contingency tables that corrected for multiple comparisons showed no confounding between order and sample type ($\chi^2 = 0.96$, $P = 0.83$; fig. S2A), while order and assay method were associated ($\chi^2 = 7.67$, $P = 0.02$); bird records were more likely to use agar plate assays, while bat records were more likely to use microplate spectrometry (fig. S2B). Order and study design were also highly associated ($\chi^2 = 18.75$, $P < 0.01$), as most bird records (17/18) but almost no bat records (1/9) experimentally altered handling or storage time (fig. S2C). Finally, while order and freezing temperature were not associated ($\chi^2 = 0.13$, $P = 1$), we highlight that proportionally more bird records (1/5) stored plasma samples above -80°C than bat records (1/8; fig. S2D).

Predictors of BKA Effect Size

Species order and total CFUs were the most important predictors of difference in BKA associated with handling or storage

time (table 1). Mean effect size was significantly different between orders after adjustment for total CFUs ($Q_2 = 9.90$), with Aves showing a significantly lower effect size than Chiroptera ($\beta_{\text{Aves}} = -0.76$, $z = 2.77$, $P < 0.01$; fig. 1). When total CFUs were held at the median, birds showed an overall negative correlation between these conditions and BKA ($r = -0.59$, 95% confidence interval [CI]: -0.81 to -0.22) while bats showed no mean effect ($r = 0.08$, 95% CI: -0.21 to 0.36). The MEM with order and total CFUs was the most competitive ($w_i = 0.26$) and explained 59% of effect size variation. Models with both these terms and either a sample volume or a BKA covariate were also competitive ($\Delta\text{AICc} = 0.49$ – 1.00 , $w_i = 0.20$ – 0.16 ; table 1), suggesting these methodological terms to be uninformative (Arnold 2010). Models with only a BKA covariate (i.e., handling time or storage time) and its interaction with species order were also not competitive ($w_i \leq 0.03$; table 1), suggesting that effect size did not substantially differ across these data subsets and thus justifying pooling both into one analysis. Our most parsimonious model (with species order and total CFUs as fixed effects) received even more support when we assigned a positive sign to the effect size where directionality was not determined and repeated the model comparison procedure ($w_i = 0.27$; $Q_2 = 10.58$, $R^2 = 62\%$, $P < 0.01$; fig. S3A). To assess whether this pattern was driven by broader methodological variation, we also performed a post hoc test in which we refitted this same MEM to the microplate assay subset of data ($n = 11$); effect sizes were similarly significantly different between bats and birds under this same model structure ($\beta_{\text{Aves}} = -1.53$, $z = 2.90$, $P < 0.01$; fig. S3B).

The relationship between order and effect size did not change when we stratified our data by BKA covariates. Likelihood ratio tests revealed that MEMs with only order had better support than MEMs that included order and potential confounder variables: maximum handling time ($\chi^2 = 1.29$, $P = 0.26$), maximum time frozen ($\chi^2 = 0.006$, $P = 0.94$), and freezing temperature ($\chi^2 = 3.20$, $P = 0.07$). Within these multivariable models, Aves still had significantly lower effect size than Chiroptera after adjustment for maximum handling time ($\beta = -0.85$, $z = 2.05$, $P = 0.04$), maximum storage time ($\beta = -0.36$, $z = 2.19$, $P = 0.03$), and freezing temperature ($\beta = -0.35$, $z = 2.31$, $P = 0.02$).

Phylogenetic Patterns

Across models, the variance components of species (σ_{species}) were typically lower than the variance components of study (σ_{study}), suggesting weak phylogenetic dependence (table 1). WPGLS models fitted with ML further showed weak phylogenetic signal in the logit proportion of nonnegative effect sizes (Pagel's $\lambda = 0.04$) but a marginally higher effect of phylogeny on the logit proportion of positive effect sizes (Pagel's $\lambda = 0.14$). WPGLS models fitted with REML that included order showed that Chiroptera had a larger proportion of logit-transformed nonnegative ($\chi^2 = 19.7$, $P < 0.001$) and positive ($\chi^2 = 350$, $P < 0.001$) effect sizes than Aves (fig. 2).

Table 1: Ranking of mixed-effects models predicting effect size for the relationship between handling or storage time and BKA for bats and birds ($n = 25$)

Fixed effects	k	σ_{species}	σ_{study}	σ_{unit}	ΔAICc	w_i	R^2
Total CFUs + species order	3	0	.093	.001	0	.26	.59
Total CFUs + species order + sample volume	4	0	.05	.001	.49	.2	.77
Total CFUs + species order + BKA covariate	4	0	.073	0	1	.16	.68
Species order	2	.004	.175	.001	2.49	.07	.2
Intercept only	1	.002	.221	.001	2.65	.07	0
Total CFUs	2	.082	.119	.001	4.02	.03	.11
Species order + BKA covariate	3	.002	.163	0	4.22	.03	.26
BKA covariate	2	.01	.205	0	4.29	.03	.04
Assay method	2	0	.238	.001	4.62	.03	0
Sample volume	2	0	.223	.001	5.29	.02	0
Sample type	2	0	.213	.001	5.56	.02	.05
Total CFUs + BKA covariate	3	.094	.092	0	5.57	.02	.17
Species order + sample volume	3	.003	.188	.001	6.03	.01	.15
BKA covariate + sample volume	3	0	.21	0	7.15	.01	.07
Total CFUs + assay method	3	0	.265	.001	7.3	.01	0
Total CFUs + sample type	3	.076	.123	.001	7.43	.01	.11
Total CFUs + sample volume	3	.076	.137	.001	7.53	.01	.05
Species order \times BKA covariate	4	.002	.201	0	7.96	0	.1
Assay method + sample volume	3	0	.244	.001	8.09	0	0
Species order + BKA covariate + sample volume	4	0	.176	0	8.1	0	.22
Sample type + sample volume	3	0	.224	.001	8.77	0	0
Total CFUs + BKA covariate + sample volume	4	.1	.098	0	9.43	0	.12
Total CFUs + assay method + sample volume	4	0	.272	.001	11.2	0	0
Total CFUs + sample type + sample volume	4	.077	.133	.001	11.33	0	.06

Note. Models are ranked by ΔAICc , with the number of parameters (k), estimated variance components (σ_i), Akaike weights (w_i), and R^2 . AICc = Akaike information criterion corrected for small sample size; BKA = bacterial killing ability; CFUs = colony-forming units.

Publication Bias

We found some evidence of publication bias in how handling or storage time affects BKA for birds and bats (fig. S4). We did not detect an association between effect size and standard error for Chiroptera ($z = 0.36$, $P = 0.72$) but did identify a marginally significant association for Aves ($z = -1.73$, $P = 0.08$). Accordingly, trim-and-fill analyses using the R_0 estimator suggested that the number of missing studies did not differ from 0 for Chiroptera (1 ± 2 , $P = 0.25$) but did differ significantly from 0 for Aves (9 ± 4 , $P < 0.01$), suggesting potential bias against publishing nonsignificant or positive correlations for birds but not for bats.

Discussion

A knowledge gap in research on emerging pathogens is whether immunological factors explain the propensity for bats to host virulent pathogens without disease (Baker et al. 2013; Brook and Dobson 2015; Plowright et al. 2016; Schountz et al. 2017). While some components of bat immune systems may differ from those of other taxa (Hatten et al. 1973; Stockmaier et al. 2015; Zhou et al. 2016; Xie et al. 2018), comparative studies across orders are rare. Comparison between the greater mouse-eared bat (*Myotis myotis*) and the house mouse (*Mus musculus*) showed that bat

macrophages challenged with toll-like receptor ligands, lipopolysaccharides, and polyinosinic-polycytidylic acid mounted an antiviral and proinflammatory response that was balanced by sustained high-level transcription of anti-inflammatory cytokines (while mice mounted only the former), suggesting the evolution of anti-inflammatory responses to neutralize effects of flight-induced metabolic stress (Kacprzyk et al. 2017). Broader comparisons across mammalian and chicken innate immune systems showed that while bat interferons are generally consistent with those of other taxa, basal transcription of antiviral interferons was higher in bats than in other taxa (Shaw et al. 2017). Our phylogenetic meta-analysis of BKA effect sizes adds to this growing literature by presenting preliminary evidence suggesting that bats and birds could likewise differ in how their constitutive innate immune defense responds to the acute stress of capture as well as the resilience of these immune components to temperature variation of storage conditions.

Specifically, models controlling for host phylogeny, sample size, and study-level covariates (i.e., total microbe CFUs) showed that species order was a key predictor of bat and bird BKA response to handling stress and storage conditions, while many methodological predictors (e.g., sample volume and type, assay type) were uninformative. The influence of order was independent of whether records assessed handling time or storage time

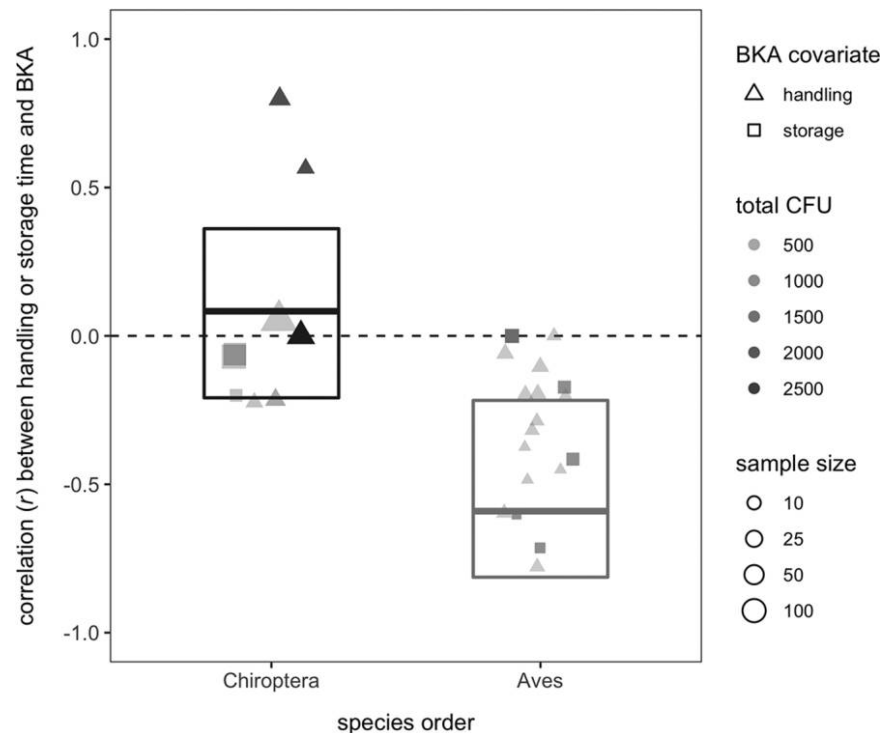


Figure 1. Correlation between handling or storage time and bacterial killing ability (BKA) with fitted mean (thick line) and 95% confidence interval for the top mixed-effects model predicting effect size by species order and total microbe colony-forming units (CFUs). Predicted values were generated by holding total CFUs at the median. Points show individual records shaped by BKA covariate, shaded by total CFUs, and sized by the sample per record. The dashed line indicates that there is no relationship between handling or storage time and BKA ($r = 0$). A color version of this figure is available online.

(i.e., the interaction between BKA covariate and order was not competitive; table 1). While these handling or storage conditions were associated with impaired BKA for birds, bat BKA appeared more resilient. A phylogenetic comparative approach further suggested that the proportions of nonnegative and positive effect sizes (e.g., handling stress and storage time had no effect or a positive effect on BKA) were also significantly higher in bats than in birds, although this analysis was limited by smaller sample sizes. While we also adjusted for potential confounding variables in our primary analyses (e.g., total microbe CFUs, sample volume) and within several specific subset analyses (e.g., refitting models to only microplate assay data, controlling for maximum handling time, maximum storage time, and freezing temperature), biases could be introduced by differences in bat and bird research methodology. Research on BKA of birds was more likely to use agar plate assays, while that on bat BKA was more likely to use microplate spectrometry. Although results from these methods are strongly correlated (French and Neuman-Lee 2012), variation in assay methodology could introduce additional noise into comparative analyses. Furthermore, most effect sizes for birds were derived from experimentally imposed conditions, whereas almost all effect sizes for bats were from observational studies. Such findings suggest an important need for standardization of assays to measure BKA across taxa and for more experimental

manipulation of stress and storage impacts on BKA in bats in particular.

Our subset analysis showed that bats and birds may differ in how BKA responds to sample storage after variation in maximum storage time and freezing temperature is accounted for. BKA against *Escherichia coli* ATCC 8739 depends primarily on complement proteins (Millet et al. 2007; Moore et al. 2011; French and Neuman-Lee 2012), suggesting potential resilience of bat complement, in particular, to sample storage duration. Previous work comparing complement levels across several mammals demonstrated that hemolysis of big brown bats (*Eptesicus fuscus*) was less sensitive to temperature change than that of guinea pigs (*Cavia porcellus*) and Malaysian flying foxes (*Pteropus vampyrus*), which suggests that proteins involved in the complement activity of the Yangochiroptera could be distinct (Hatten et al. 1973). Functional enzyme activity over a broader temperature range may be a biological necessity in species for which body temperatures can vary extensively. For example, BKA of snapping turtles (*Chelydra serpentina*) also was not affected by 6 wk of storage at -80°C (Beck et al. 2017), and little brown myotis (*Myotis lucifugus*) were able to clear *E. coli* throughout their hibernation period (Moore et al. 2011). Only two of the included bat species hibernate (*Tadarida brasiliensis* and *Nyctalus noctula*), and therefore we were unable to assess intraorder differ-

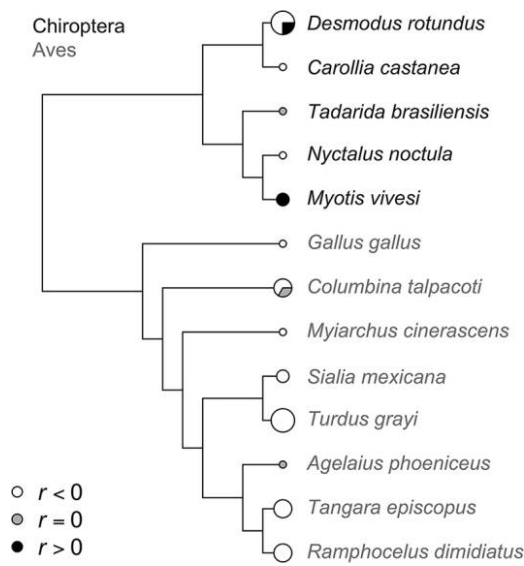


Figure 2. Phylogenetic tree of bat and bird species included in the phylogenetic meta-analysis, with tips colored by species order. Circles display the proportion of negative, null, or positive relationships between handling or storage time and BKA and are scaled by the sample size. A color version of this figure is available online.

ences in the BKA response to storage time between hibernating and nonhibernating species. However, this result should encourage future comparative studies of the temperature dependence of complement between hibernating and nonhibernating species of bats and rodents to assess whether torpor is associated with the evolution of enzyme resiliency pertinent to antibacterial defenses. While we restricted our analysis to small-bodied endotherms to control for body mass and evolutionary history, broader comparative analyses could also assess whether this result represents a difference between bats and birds or between birds and mammals more generally, as a similar resilience of BKA to storage has been reported for some carnivores (Flies et al. 2016; Heinrich et al. 2016).

Bats and birds also showed a strong difference in the resilience of BKA to the acute stress of capture. Both acute and chronic stress can negatively influence host immunity (Padgett and Glaser 2003), although species can show different physiological responses to stress that affect these immunological outcomes (Korte et al. 2005). Bat BKA had no association with handling time, even after variation in the maximum minutes between capture and sampling across studies was accounted for, while bird BKA showed an overall negative relationship. Acute capture stress in little brown bats (*M. lucifugus*) and Malaysian flying foxes (*P. vampyrus*) was associated with 15–60 min of elevated circulation of glucocorticoids (Reeder et al. 2004, 2006); therefore, it is unlikely that our results stem from a negligible stress responses in bats. One explanation could be that the energetic cost of complement activity is relatively low, compared to that of other immune mechanisms (e.g., induced cell-mediated responses; Lee 2006); bats might therefore prioritize complement immunity as

part of their physiological adaptations to buffer metabolic costs of flight (O'Shea et al. 2014; Brook and Dobson 2015). The energetically cheaper complement system may provide bats with higher BKA that is relatively refractory to the impacts of acute stress (Matson et al. 2006). Additional experimental tests of how acute and chronic stress affect BKA more broadly across the Chiroptera, as performed for birds (Matson et al. 2006; Zylberberg 2015) but only one bat species (Strobel et al. 2015), would provide further insights into potential differences in the bat complement system compared to those of other host taxa.

Flight as an underlying mechanism for the potential resilience of bat complement to stress could be further tested by comparing the responses of BKA not only between volant and nonvolant small mammals (i.e., Chiroptera and Rodentia) but also between volant and nonvolant birds. Flightlessness likely evolves in the absence of avian and mammalian predators and in response to restricted resource supply, resulting in smaller flight muscles and rates of energy expenditure (McNab 1994; Wright et al. 2016). While comparisons of effect size between flightless birds and those capable of flight could provide complementary insights to comparisons between volant and nonvolant mammals, only one species within our data set is flightless (*Gallus gallus*). It also remains unknown whether immunological mechanisms associated with the evolution of flight (e.g., bats) would be lost with the evolution of flightlessness (e.g., in nonvolant birds). Additional empirical studies of how stress affects complement-mediated defense more broadly across taxa could facilitate more robust comparative tests of flight as a driver of the patterns observed here.

More broadly, mammals and birds show several key differences in their complement systems, with Aves having fewer components, receptors, and regulatory factors than the Mammalia in particular (Nakao and Somamoto 2016). Formation of the lytic membrane attack complex (MAC) in the vertebrate complement cascade can be activated in a number of ways that require formation of antigen-antibody interactions (classical pathway), microbe carbohydrate recognition by mannan-binding lectin (lectin pathway), or an alternative pathway that is activated by most foreign antigens in the absence of specific antibodies. While these three pathways rely on activation of C3 convertase, activation of anaphylatoxin and opsonin (C3a, C5a) and MAC (C5b–C9) by a fourth pathway is mediated by thrombin (Huber-Lang et al. 2006). Birds lack a protease (MASP1-specific serine protease) involved in both the lectin pathway and that is also critical for triggering the alternative pathway (Lynch et al. 2005). Aves also appear to lack C6, C7, C8, and C9, which form the MAC (Nakao and Somamoto 2016), but these are also understudied in birds, compared to fish, amphibians, and mammals (Dodds and Matsushita 2007).

Depletion of the complement system would facilitate explicitly assessing differences in complement and its role in BKA between bats, birds, and rodents, although we were unable to assess comparisons with the latter taxon or with flightless birds, because of data availability. While heat inactivation of plasma mostly eliminated BKA of big brown bats (*E. fuscus*; Moore et al. 2011), complement of other species may be less sensitive to temperature changes (Hatten et al. 1973), thereby rendering the

standard heat-inactivation process less effective. Alternatively and pending reagent availability, other methods of complement inactivation could be employed, including chemical methods (Ecker et al. 1938), plasmin-mediated complement depletion (Pillemer et al. 1953), and inhibition of selective steps in the complement cascade (Noris and Remuzzi 2013; Johnson et al. 2015). While the complement system is an important component of plasma, other plasma proteins, including immunoglobulins, fibrinogen, and thrombin (clotting factors), and albumins may also contribute to potential differences in BKA between orders, either by supporting complement activation or by expanding the antimicrobial capacity of innate cells (Delvaeye and Conway 2009; Noris and Remuzzi 2013). To determine which complement pathway (i.e., classical, lectin, or alternative) could be involved in the potentially more resilient BKA of bats, plasma could be treated to selectively deplete these proteins (Jenkins et al. 2008; Selvaraju and El Rassi 2012; Uzun et al. 2013). To determine whether clotting factor is involved in bat BKA and differs between taxa, serum could be used instead of plasma. Finally, because complement also enhances antimicrobial functions of leukocytes, future work could also assess whether antibacterial defense by neutrophils and macrophages is stronger in bats than in other taxa.

While our phylogenetic meta-analysis suggests that bat BKA may be more resilient to acute stress and storage time than volant-bird BKA, this finding should not be interpreted as permitting leniency in how bat samples are analyzed for ecoimmunological studies. Researchers should continue to record relevant covariates such as handling time, storage time, and freezing temperature so that statistical analyses can control for such effects (Moore et al. 2011; Becker et al. 2018b). We also identified a potential preference to publish negative effect sizes (e.g., $r < 0$) for Aves but not for Chiroptera. In addition, our literature search identified no studies that reported the influence of handling or storage time on BKA in Rodentia, despite identifying an equal number of BKA studies for both Rodentia and Chiroptera. The improved reporting of covariates and effect sizes more generally across these host taxa would facilitate more robust comparative insights into how BKA responds to acute stress and changes in temperature and whether relationships vary for defenses against other pathogens (e.g., Gram-positive bacteria and fungi), especially given the small sample sizes included in our analyses here and risks of Type II errors.

We therefore hope that our preliminary analysis will prompt future comparative studies on innate immune defense to elucidate mechanisms driving the results we report here, especially as immune response to extracellular bacterial pathogens may not reflect immune defense against intracellular viruses, bacteria, and protozoa (Brook and Dobson 2015). Future studies that can compare multiple measures of immunity across sympatric bats, birds, and rodents (to control for environment), using both classic ecoimmunology techniques and more comprehensive RNA sequencing methodology (Demas et al. 2011; Fassbinder-Orth 2014), will be critical to identify the aspects of vertebrate immune defense unique to flight, hibernation, and the Chiroptera in particular.

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