Body size affects immune cell proportions in birds and non-volant mammals, but not bats

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Summary statement: Powered flight might constrain morphology such that certain immunological features are prioritized. We show that bats largely have similar cell proportions across body mass compared to strong allometric scaling relationships in birds and non-flying mammals.

Abstract

Powered flight has evolved several times in vertebrates and constrains morphology and physiology in ways that likely have shaped how organisms cope with infections. Some of these constraints likely have impacts on aspects of immunology, such that larger fliers might prioritize risk reduction and safety. Addressing how the evolution of flight may have driven relationships between body size and immunity could be particularly informative for understanding the propensity of some taxa to harbor many virulent and sometimes zoonotic pathogens without showing clinical disease. Here, we used a scaling framework to quantify scaling relationships between body mass and the proportions of two types of white blood cells--lymphocytes, and granulocytes (neutr-/heterophils)--across 60 bat species, 414 bird species, and 256 non-volant mammal species. By using phylogenetically-informed statistical models on field-collected data from wild Neotropical bats, data gleaned from other wild bats available in the literature, and data from captive non-volant mammals and birds, we show that lymphocyte and neutrophil proportions do not vary systematically with body mass among bats. In contrast, larger birds and non-volant mammals have disproportionately higher granulocyte proportions than expected for their body size. Future comparative studies of wild bats, birds, and non-volant mammals of similar body mass should aim to further differentiate evolutionary effects and other aspects of life history on immune defense.

Introduction

Powered flight has evolved at least three times in the evolutionary history of vertebrates and yet is one of the most energetically costly modes of transportation (Rayner, 1988). Birds and bats experience a 6–14 fold and >25 fold increase over resting metabolic rate, respectively, in metabolic expenditure during flight, whereas a similarly-sized mammal only experiences a 6–8 fold increase during sustained running (Schmidt-Nielsen, 1972; Thomas, 1975). Although there is some debate over whether bats or birds are more efficient fliers (Muijres, Johansson, Bowlin, Winter, and Hedenström, 2012; Swartz et al., 2007; Tian et al., 2006), there are clear functional and physiological constraints associated with this costly activity (Maurer et al., 2004; Muijres et al., 2012). One of the most evident constraints is body size. Exceptionally large and small body sizes have apparently been selected against in the evolution of flying vertebrates due to demands imposed by the physics of flight (Stanley, 1973); however, the constraining factors for bats and birds likely differ, as the largest bats are much smaller than the largest flying birds. The evolution of flight and body size constraints may have had numerous direct and indirect effects on evolution of the immune system in flying vertebrates. For example, evolution of a lightened skeleton (Feduccia and Feduccia, 1999; Dumont, 2010) may affect how immune cells are differentiated and distributed throughout the body. If larger fliers are not as efficient at circulating protective cells throughout their bodies, then they might require greater quantities of cells (Ruhs et al., 2020). It should be noted that the high energetic costs of flight have varying impacts on the immune system (Hasselquist et al. 2007; Voigt et al. 2020; Nebel et al. 2012). While birds and bats have much in common in terms of constraints that accommodate the ability to fly, the evolution of flight likely impacted the dynamics between body size, physiological traits, and the exposure risk to pathogens relative to non-flying birds and mammals.

Body size influences almost all life processes and structures of organisms (Brown, Gillooly et al. 2004; West et al. 2000). Many biological traits vary with body size in predictable ways; some vary proportionally across body size (i.e., isometric scaling), whereas others change disproportionately with size (i.e., hyper or hypometric scaling; Calder, 1996; Kleiber and Others, 1932; Knut Schmidt-Nielsen and Knut, 1984). Most efforts to describe relationships between size and traits take the form:

$$Y=aM^b \quad \text{or} \quad \log(Y)=\log(a) + b*\log(M)$$

where (in the linearized form) b represents the scaling coefficient, M is body mass, a denotes the intercept, and Y represents the trait of interest. Many traits influenced by body size, including lifespan and movement patterns (e.g., home range size, distance traveled while foraging), affect pathogen exposure (Han et al., 2015), which could in turn exert selective pressure on how species allocate resources to immune defense (Brace et al., 2017; Lee, 2006).

Although various hypotheses predict distinct forms of scaling for aspects of immunity (Cohn and Langman, 1990; Dingli and Pacheco, 2006; Wiegand and Perelson, 2004), there is strong evidence that particular immune cells (namely concentrations of granulocytes, such as the neutrophils of mammals (Downs et al., 2020) and heterophils of birds (Ruhs et al. 2020) scale hypermetrically with body size. These allometries support the Safety Factor Hypothesis, which proposes that larger animals favor infection risk reduction by investing heavily in safety (Downs et al., 2020; Harrison, 2017), in particular by using a reserve pool of broadly protective granulocytes (e.g., neutrophils and heterophils). However, concentrations of heterophils in birds scale at a steeper rate ($b=0.19$; Ruhs et al., 2020) than mammals ($b=0.11$; Downs et al., 2020). Although this difference could be an evolutionary artifact or driven by one of many other differences between birds and mammals, this steep allometry has been hypothesized to be related to flight, which may put larger birds at a higher risk of parasite exposure (Downs et al., 2019; Ruhs et al., 2020). It should also be noted that larger birds and bats also have longer lifespans than similarly sized non-volant mammals (Munshi-South and Wilkinson, 2010; Wilkinson and Adams, 2019), possibly putting them at increased risk of infection over their long lifespans. The potential for larger fliers to prioritize risk-reduction immunological strategies motivates our interest to investigate immune scaling in bats and among bats, birds, and other mammals.

Bats are a hyperdiverse taxon (Order Chiroptera, over 1400 species) with a nearly global distribution across habitats ranging from rainforests to deserts (Gunnell and Simmons, 2012; Simmons and Cirranello, 2020). Their unique habits and life histories (e.g., powered flight, echolocation, long lifespans despite small body sizes) make bats a notable taxon for basic studies of ecology and evolution (Ingala et al., 2018; Jones and Teeling, 2006; Wilkinson and South, 2002). Bats have also been increasingly studied for their ability to harbour some viruses that are detrimental and often lethal to humans and domestic animals (Brook and Dobson, 2015; Guth et al., 2019). Bats are confirmed reservoir hosts for henipaviruses, Marburg virus, various lyssaviruses, and most SARS-like coronaviruses (Amman et al., 2015; Banyard et al., 2011;

Halpin et al., 2011; Li et al., 2005). Yet with some exceptions (e.g., *Rabies lyssavirus*), these viruses appear to not kill and rarely cause clinical disease in bats (Williamson et al., 2000).

Whereas the high diversity of zoonotic viruses in Chiroptera might be partly driven by the speciose nature of this order (Mollentze and Streicker, 2020), bat tolerance of particular viruses may be shaped by specialized immune mechanisms in these flying mammals (Brook et al., 2020; Zhang et al., 2013). Bat immunoglobulins and leukocytes are structurally similar to those of humans and mice (Baker et al. 2013), but bats also have unique immune system traits such as complement proteins robust to temperature change, lack of fever with bacterial (lipopolysaccharide) challenge, high constitutive expression of type I interferons, and dampened inflammation (Ahn et al., 2019; Hatten et al., 1973; Pavlovich et al., 2018; Stockmaier et al., 2015; Zhou et al., 2016). Flight may explain these distinctions, including increased metabolic rates that enable stronger immune responses and elevated body temperature that could mirror febrile responses to control infection (O'Shea et al. 2014; but see Levesque et al. 2020). However, the primary hypothesis for how bats can tolerate viruses is that they evolved mechanisms to minimize or repair the negative effects of oxidative stress generated as a consequence of flight (Zhang et al., 2013). For example, some bat species show resistance to protein oxidation and unfolding (Salmon et al. 2009), reduced lipid peroxidation (Wilhelm Filho et al. 2007), and lower hydrogen peroxide per unit oxygen consumed (Brunet-Rossinni 2004). This propensity to resist acute oxidative stress and repair oxidative damage could have also helped bats cope with viral replication that would have otherwise caused cell damage (Kacprzyk et al., 2017; Xie et al., 2018; Zhang et al., 2013).

Here, we first asked whether leukocyte proportion scaling in bats is distinct compared to other taxa already described. Then, we asked whether the ability to fly (i.e. bats and birds) explains immune cell proportion allometries across extant vertebrate endotherms. Most studies assessing immunity in bats have been limited to few species (but see Schneeberger et al., 2013). We combined field-collected data from Neotropical bats with data from the primary literature to maximize sample sizes as well as phylogenetic and body size diversity. We then quantified scaling relationships for proportions of two primary leukocytes for which abundant data were available, lymphocytes and granulocytes. Lymphocytes include B and T cells, which provide specific, but time-lagged, protection through antibody production and coordination of cascading immune responses. Granulocytes (neutrophils in mammals and heterophils in birds) are

phagocytes that rapidly protect against micro- and macroparasites without education or much specificity (Lanier, 2013). Finally, we directly compared scaling relationships for cell proportions in bats to those of birds and non-volant mammals using an existing database (ZIMS).

We predicted the forms of relationships between cell proportions and body size based on previously discovered scaling patterns among body size and cell concentrations (Downs et al., 2020; Ruhs et al., 2020). Proportions, however, sometimes pose difficulties for studies of allometry because they are bound rather than having no continuous upper limit and are inherently co-dependent (i.e. one cell type goes up, another goes down). As leukocyte concentration data for bats are extremely rare in the literature, and proportional data permitted comparisons that were otherwise presently impossible, we estimated scaling patterns using proportional data but encourage caution in comparing results from this analysis against prior scaling for leukocyte concentrations. We hypothesized isometry for lymphocytes in bats, as was observed previously for bird cell concentrations (Ruhs et al., 2020). We expected isometry to manifest because lymphocytes are a functionally diverse group of cells including both B and T cells (Lanier, 2013), the proportions of which could vary dramatically among species. By contrast, granulocyte functions are fairly homogeneous, so we predicted hypermetric granulocyte scaling in bats as was observed in other mammals and birds (Downs et al., 2020; Ruhs et al., 2020). However, as larger fliers might overinvest in safety, we expected bat neutrophil proportions to scale hypermetrically, to the same degree (steeper than non-volant mammals) as was observed for heterophil concentrations in birds (predictions based on if flight influences scaling; Fig. 1; Ruhs et al., 2020). Alternatively, we could observe no impacts of flight on cell proportion allometries, which could be due to life-history features (e.g. reproduction, sociality) or equal investment in risk reduction strategy due to factors like increased lifespan, regardless of body size.

Materials and methods

Bat sampling

During April and May in 2017 and 2018, we sampled 160 bats from 26 species in the Orange Walk District of Belize (Herrera et al., 2018). Bats were captured using mist nets and harp traps and were identified to species based on morphology (Reid, 1997). We collected blood by lancing the propatagial vein with a sterile needle, followed by collection using heparinized capillary tubes. Thin blood smears were prepared and stained with buffered Wright–Giemsa (Astral

Diagnostics Quick III). All bats were released after processing. Sampling followed guidelines for safe and humane handling of bats from the American Society of Mammalogists (Sikes and Gannon, 2011) and was approved by the Institutional Animal Care and Use Committees of the University of Georgia (A2014 04-016-Y3-A5) and American Museum of Natural History (AMNH IACUC-20170403). Sampling was authorized by the Belize Forest Department under permits WL/2/1/17(16), WL/2/1/17(19), WL/2/1/18(16).

Bat leukocyte data

We used light microscopy (1000X) to quantify the proportion of neutrophils and lymphocytes from 100 leukocytes from each field sample (Schneeberger et al., 2013). As Neotropical bats are relatively limited in their range of body masses, we supplemented our leukocyte dataset with a systematic literature search (Fig. S1). We identified articles using Web of Science and the search terms TS=(bat OR Chiroptera OR flying fox) AND (hematology OR white blood cell OR leukocyte). For bat species sampled across multiple studies, we averaged cell proportions. When available, body masses of each bat species were extracted from EltonTraits (Wilman et al., 2014); however, for a few species ($n=2$), masses were averaged from the source paper. The literature search substantially increased our body mass range (from approximately 5-78 grams to 4-804 grams; see Figure S2 for a comparison across all extant bat species; Wilman et al., 2014). Within-species sample size ranged from 1 to 140 ($\bar{x}=15 \pm 3$) but did not predict proportions of either cell type (neutrophils: $\rho=-0.09$, $p=0.48$; lymphocytes: $\rho=0.06$, $p=0.67$).

Bird and non-volant mammal data

To compare bat leukocyte data to comparable data from birds and non-volant mammals, we extracted species means of lymphocyte and granulocytes (neutr-/heterophils) proportions in whole blood from ZIMS (Species360, 2019). ZIMS is a repository of veterinary data from captive, adult animals housed in facilities accredited by the Association of Zoos and Aquariums and considered healthy. We removed bat data ($n=3$) from the extracted ZIMS mammal database. When cleaning the data, we only included data from Global Species Reference Intervals. We compiled standardized species-level body mass data from the CRC Handbook of Avian Masses (Dunning Jr., 2007) and/or publicly available databases such as AnAge (Taddei et al., 2013), the Animal Diversity Website (Jones et al., 1997), and the Encyclopedia of Life (Parr et al., 2014).

Statistical analyses

Exercise 1: best-fit models for leukocyte proportion allometries in birds, bats, and non-volant mammals

Our modeling progressed in two stages. First, to test hypotheses about allometric scaling of leukocytes in bats only, we used phylogenetic generalized mixed-effects models (GLMMs) with the *ape* and *MCMCglmm* packages in R (Hadfield & Others, 2010; Paradis et al., 2004). All models included phylogenetic effects from a phylogeny produced in PhyloT using data from the National Center for Biotechnology Information (Letunic, 2015) and with resolved polytomies. We used that tree to create two phylogenetic covariance matrices, one for bat-only analyses and one that we used later for direct comparisons of scaling slopes across taxa. We set the inverse-gamma priors to 0.01 for the random effect of phylogenetic variance and default priors for the fixed effects in all models. All models were run for 260k iterations with 60k burn-in and a 200-iteration thinning interval (Downs et al., 2020; Ruhs et al., 2020). For all models, we used Deviance Information Criterion (Δ DIC) to identify the best-fit GLMM. We defined the top model as that with the lowest DIC, and we considered models within Δ DIC<5 as having equivalent support (Richards, 2005). For all models, we also calculated Pagel's unadjusted λ and conditional and marginal R^2 (Housworth et al., 2004; Nakagawa and Schielzeth, 2013). We then used this approach to determine the scaling relationship for lymphocyte and neutrophil proportions, separately, across 60 bat species. Also, because previously published slopes for mammal and bird leukocyte scaling used cell concentrations (Downs et al., 2020; Ruhs et al., 2020), we determined the scaling relationships of cell proportion data for both birds ($n=414$) and mammals ($n=256$) independently to facilitate direct comparisons with bats. Results from bird and mammal-only models are presented in the online supplement (Tables S1 and S2). For each taxonomic group and each cell type, we produced two sets of *a priori* models:

$$\text{Model 1. } \log_{10}(\text{leukocyte proportion}) = \log_{10}(a) + \varepsilon$$

$$\text{Model 2. } \log_{10}(\text{leukocyte proportion}) = \log_{10}(a) + b * \log_{10}(\text{body mass}) + \varepsilon$$

Model 1 represents a null model with $b=0$, whereas model 2 estimates the scaling relationship between body mass and cell proportion.

Exercise 2: direct comparisons of allometries among taxa

Next, we directly compared the slopes of relationships between body mass and immune cell type in bats ($n=60$), birds ($n=414$), and non-flying mammals ($n=256$). Specifically, we fit five models to the data and compared DIC scores to determine the best-fit versions

$$\textbf{Model 3. } \log_{10}(\text{leukocyte proportion}) = \log_{10}(a) + \varepsilon$$

$$\textbf{Model 4. } \log_{10}(\text{leukocyte proportion}) = \log_{10}(a) + b * \text{taxon} + \varepsilon$$

$$\textbf{Model 5. } \log_{10}(\text{leukocyte proportion}) = \log_{10}(a) + b * \log_{10}(\text{body mass}) + \varepsilon$$

$$\textbf{Model 6. } \log_{10}(\text{leukocyte proportion}) = \log_{10}(a) + b_1 * \text{taxon} + b_2 * \log_{10}(\text{body mass}) + \varepsilon$$

$$\textbf{Model 7. } \log_{10}(\text{leukocyte proportion}) = \log_{10}(a) + b_1 * \text{taxon} + b_2 * \log_{10}(\text{body mass}) + b_3 * \log_{10}(\text{body mass}) * \text{taxon} + \varepsilon$$

Here, model 3 represents a null model with $b=0$, and model 4 only tests for mean differences in cell proportions per taxon (i.e., bat, bird, non-volant mammal), irrespective of body mass. Model 5 is analogous to model 2 from exercise 1, and it estimates a global scaling relationship between body mass and cell proportions across all taxa. Lastly, model 6 combines models 4 and 5 (i.e., mean differences between taxa and a global body mass slope), whereas model 7 explicitly tests whether scaling relationships between body mass and cell proportions differ among taxa.

Results

Exercise 1: best-fit models for leukocyte proportion allometries in birds, bats, and non-volant mammals

For bats, the intercept-only model (fitting $b=0$; model 1) and the mass model (model 2) received equivalent support for both lymphocytes and neutrophils (Table 1, Fig. 2). Slopes for lymphocytes (b , CI=-0.08, -0.24:0.08) and neutrophils (b , CI=0.06, -0.09:0.22) were indistinguishable from zero (Table S1, Figs. 2 and 3). Although these results suggest isometry for lymphocytes and neutrophils, we encourage caution in their interpretation given that null models can represent either a true slope of zero or a lack of power to find allometry. For lymphocytes, phylogeny accounted for very little of the variation (16%) and the addition of body mass increased explanatory power by 15%. For neutrophils, again, phylogeny accounted for very

little of the variation (21%), and the addition of body mass increased explanatory power by only 4%. For both leukocyte types, the model fit of the mass model was 33-37% and did not increase much from the 19-29% of the intercept-only model. Collectively, our results suggest little allometric scaling of proportions of either cell type among bat species.

For birds, the mass model (model 2) was best-supported (Table S2) for both cell types; lymphocytes scaled hypometrically (b , CI=-0.09, -0.1:-0.08; Table S3; Fig. 3) and heterophils scaled hypermetrically (b , CI=0.09, 0.08:0.11; Table S3). For non-volant mammals, the lymphocyte and neutrophil mass models were also the best-supported (Table S4); lymphocytes scaled hypometrically (b , CI=-0.08, -0.1:-0.06; Table S5; Fig. 3) and neutrophils scaled hypermetrically (b , CI=0.04, 0.03:0.06; Table S5). Phylogeny explained between 63-68% of the variation in birds and 70-79% of the variation in mammals (Tables S2, S4). Bird and mammal granulocytes and mammalian lymphocyte cell proportion scaling patterns were consistent in direction (but not magnitude) with previous analyses of cell concentrations (Downs et al., 2020; Ruhs et al., 2020). However, bird lymphocyte proportions were hypometric here whereas no evidence of allometry in lymphocyte concentrations was previously reported (Ruhs et al., 2020).

Exercise 2: direct comparisons of allometries among taxa

When bat lymphocyte proportions were directly compared to those of birds and non-volant mammals, we found equivalent support for the mass model (model 5; Table 2, Fig. 2), the model with independent effects of mass and taxon (model 6; Δ DIC=0.67), and the model in which allometries differed between taxa (model 7; Δ DIC=3.02). For the mass model (model 5), phylogeny explained a large proportion of the variance (~70%), but the addition of mass increased explanatory power by 16% (marginal R^2). For model 6, the addition of mass and taxon increased explanatory power by an additional 13% (marginal R^2). When examining models that compared the lymphocytes among taxa (models 3, 4, 6, 7), bats were different from non-volant mammals but not birds (Table S6). Because the slope for bat lymphocyte proportions and body mass was indistinguishable from zero (from model 5, b , CI=-0.08, -0.09:-0.07; exercise 1), this effect was largely driven by differences in the intercept between taxa (-0.3 in bats; -0.14 in birds; -0.09 in mammals). In other words, mean lymphocyte proportions across all body sizes were lower in bats than other mammals.

Granulocyte proportions, however, were best explained by the model in which intercepts of allometries varied among taxa (model 7); however, this was driven by a universal hypermetric scaling slope across all species (b , CI= 0.06, 0.05:07). When examining models comparing granulocyte proportions among taxa (models 3, 4, 6, 7), bat slopes and intercepts were not different from non-volant mammals or birds (Table S7). Phylogeny explained 70% of the total variation. Inclusion of mass and taxa increased explanatory power by 11% (marginal R^2). Therefore, phylogeny explains the majority of the variance in the proportions of both cell types, but body mass informs a moderate percentage of this variation, especially compared to taxon alone (marginal $R^2=0.03$).

Discussion

Although bats and birds represent two independent evolutionary origins of flight (Rayner, 1988), both are flying endotherms. We therefore predicted they would be subject to similar selective pressures on their physiology that would ultimately impact their immune system (McGuire and Guglielmo, 2009). Here, we quantified the scaling relationships for proportions of two leukocyte types across 60 species of bats and compared these patterns across other vertebrate endotherms. Broad, comparative analyses of immunity between bats and other taxa are rare (Becker et al., 2019; Shaw et al., 2017), and allometric studies provide a powerful framework for systematically comparing such data across species (Downs et al., 2020, Ruhs et al., 2020). Therefore, our analyses aimed to shed light on bat cellular immunity, generally, and the role of flight in shaping immune scaling relationships. When we examined body mass effects on bat cell proportions (posterior means from exercise 1; Fig. 2), we found little evidence for allometric scaling of either cell type across bat species. When comparing across taxa (exercise 2), however, bat lymphocyte proportions (represented by the intercept) more closely resembled those of birds than non-volant mammals, as the confidence intervals for bats overlapped with those for birds, but not for non-volant mammals. However, bat neutrophil proportions were not distinguishable from birds or non-volant mammals, and all taxa tended to scale hypermetrically. Therefore, our results support the idea that physiological alterations to facilitate flight may not explain the allometry of cell proportions in bats and birds. Our inability to comparatively distinguish bats from other endotherms in exercise 2 is likely driven by the large amount of variation in the bat data, which are from wild populations, and thus complicates identifying allometric patterns; however, as the

diversity of leukocyte data from captive bats are low, this comparative analysis can still shed light on flight in shaping immune scaling patterns and help generate predictions for future comparisons. Below we focus on the results from exercise 1 to discuss scaling patterns for each taxon and the (likely) lack of allometry in bats. We then address immunological similarities between bats, birds, and non-volant mammals and what this might mean for pathogen tolerance to motivate future comparative research.

Are bats immunologically different?

We predicted that bats, like birds, might need a disproportionately large amount of broadly protective cells as they are more likely to be exposed to more and diverse parasites than non-volant species, especially at larger body sizes. However, we found little evidence for intercept or slope differences between bats and non-volant species or allometric scaling in cell proportions of bats. Interestingly, while bats did not show any allometries when analyzed alone (exercise 1), in exercise 2, where cell proportions were compared across taxa, bat lymphocyte proportions more closely resembled birds than non-volant mammals (taxa models panel; Fig. 2). The (likely) lack of scaling, or possible isometry, in bats is intriguing given the evident allometric scaling patterns observed previously for birds and across primarily terrestrial mammals, both for cell concentrations and the proportions analyzed here (Ruhs et al., 2020).

Importantly, contrasting leukocyte patterns between bats and other taxa could be influenced by the bat data being from wild populations and the bird/mammal data being from captive populations. Wild populations are inherently more immunologically variable (Viney & Riley, 2014), which is reflected in the large confidence intervals around our posterior mean estimates in bats. Many factors can influence wild-derived variation, but wild and captive animals can especially differ in pathogen exposure and stressors that can affect leukocyte composition (Davis et al., 2008; Herrera et al., 2019). Alternatively, the absence of allometry in our bat data (when analyzed alone in exercise 1), which are from wild populations, might be more likely to reflect true developmental and environmental pressures on these species. In fact, another study demonstrated that the variation in cell proportions from wild species did not influence scaling patterns, as wild and captive birds both displayed similar allometries (Ruhs et al., *unpublished data*). Similarly, comparison of wild and captive rodents also revealed no differences between scaling patterns of total leukocytes or neutrophil counts, despite captive

animals having higher mean lymphocyte counts than wild animals (Tian et al., 2015). Taken in sum, the lack of scaling patterns found here are unlikely driven by variation in wild bat cell proportions.

The small differences in bat versus other taxa cell proportion allometries support similar efforts to understand constitutive expression of other aspects of the bat immune system. For example, comparative genomic analyses show several unusual immunological aspects of bats, such as high constitutive expression of type I interferons and dampened inflammation (Ahn et al., 2019; Pavlovich et al., 2018; Zhou et al., 2016). Although our results are limited to proportions of two key white blood cells, the inability to distinguish bats from birds (for lymphocytes) and bats from all other taxa (for neutrophils) suggest there may be other ecological explanations (e.g. not flight-related) for the cell proportion scaling patterns.

Given the results from exercise 1, it is instead possible that the bat data are representative of isometry, such that species require the same proportions of leukocytes across body size. Isometry could be driven by certain distinct aspects of bat biology. A plausible explanation could involve their slow life-history strategy. Bats, even more so than birds, are long-lived such that selection for safety and disease risk-reduction is likely prioritized to accommodate longevity, across all body masses. Although body mass affects longevity in bats, other factors such as hibernation, cave use, and latitude all have effects of similar magnitude on lifespan as mass (Wilkinson and Adams 2019), which may complicate detecting mass effects on physiology or morphology linked to lifespan. For example, high sociality and gregariousness of many bat species facilitates contact during roosting that could increase pathogen exposure (Kerth, 2008; Kunz, 1982; Webber et al., 2017) and would be equal across body size. Although it is most likely that the bat data here represent a lack of scaling, these alternative explanations support the possibility that our bat data might represent isometry of lymphocytes when directly comparing bats to other taxa.

Future directions

Increased spillover of zoonotic viruses, such as henipaviruses and coronaviruses, has renewed public and scientific interest in whether bats are immunologically unique reservoir hosts (Brook and Dobson, 2015; Halpin et al., 2011; Li et al., 2005; Luis et al., 2013). Investigating allometric scaling patterns of immunological features, such as leukocytes, could shed light on the

physiological traits that impact host ability to tolerate virulent pathogens. We here demonstrate some differences in the scaling patterns of innate immune cell proportions between taxa of endotherms; however, we did not observe substantial effects of body size on cell proportions in bats. It is important to note that it is likely more difficult to find allometric patterns of cell proportions using the methods employed here compared to the previous discovery of hypermetric scaling of cell concentrations (Downs et al., 2020; Ruhs et al., 2020); therefore, future studies should aim to measure cell concentrations, which are much more insightful for immunological patterns. Our sample also represents only a small fraction of bat diversity (about 4% of the >1400 species; Simmons and Cirranello, 2020), although our data do span the body mass continuum of extant bat species. To enhance our ability to examine relevant patterns, we encourage greater breadth of immunological studies across the bat phylogeny, specifically within the bat clades characterized by relatively larger body sizes (e.g., Pteropodidae).

Lastly, we focused on cell proportion allometry and the potential for body mass alone to explain immunological differences among species (Downs et al., 2019). Flying endotherms can vary in other ecological traits besides body mass that also shape pathogen exposure and immune investment, such as diet, coloniality, and roost type (Minias, Whittingham, & Dunn, 2017; Schneeberger et al., 2013). To address such trait comparisons across equal ecological context (i.e., avoiding captive-wild contrasts), future comparative studies of wild bats, birds, and non-volant mammals of similar body masses could help to confirm the patterns observed here and further differentiate evolutionary effects from those of flight and other aspects of life history on immune defense.

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Data accessibility: Bat leukocyte data and endotherm metadata will be deposited in Dryad Digital Repository. Captive bird and mammal white blood cell data are available from Species360.

Table 1. Best-fit models predicting circulating leukocyte concentrations in 60 species of bats (exercise 1). Models test for the effects of body mass on log₁₀-transformed lymphocyte and neutrophil proportions. For all models, we calculated (1) Pagel's unadjusted lambda to determine the variation explained by the phylogeny not accounting for fixed effects, (2) marginal R² values to determine how much variation in leukocyte concentrations was explained by fixed effects and (3) conditional R² for overall model fit.

Model	DIC	ΔDIC	λ (unadjusted) [95% CI]	Marginal R ² [95% CI]	Conditional R ² [95% CI]
<u>Lymphocytes</u>					
1. β ₀	4.12	0.15	0.19 [0.04-0.62]		0.19 [0.04:0.62]
2. β ₀ + β ₁ x log ₁₀ (Mass)	3.96	0	0.16 [0.06:0.63]	0.002 [2.1e-6:0.17]	0.33 [0.07:0.68]
<u>Neutrophils</u>					
1. β ₀	-5.88	0	0.29 [0.07-0.68]		0.29 [0.07:0.68]
2. β ₀ + β ₁ x log ₁₀ (Mass)	-5.28	0.6	0.21 [0.06:0.69]	0.001 [1.2e-7:0.15]	0.37 [0.09:0.72]

Table 2. Best-fit models predicting circulating leukocyte concentrations in all species (bats, birds and non-volant mammals). Models test for the effects of body mass and taxon on $\log_{10}$ -transformed lymphocyte and granulocyte proportions (exercise 2). Top models are bolded.

Model	DIC	Δ DIC	λ (unadjusted) [95% CI]	Marginal R^2 [95% CI]	Conditional R^2 [95% CI]
<u>Lymphocytes</u>					
3. β_0	-1163.31	96.62	0.86 [0.78-0.92]		0.86 [0.78:0.92]
4. $\beta_0 + \beta_1 \times \text{taxon}$	-1164.48	95.45	0.83 [0.42:0.92]	0.005 [0.0001:0.5]	0.89 [0.81:0.95]
5. $\beta_0 + \beta_1 \times \log_{10}(\text{mass})$	-1259.93	0	0.7 [0.56:0.79]	0.16 [0.09:0.22]	0.84 [0.77:0.9]
6. $\beta_0 + \beta_1 \times \log_{10}(\text{mass})$ + $\beta_2 \times \text{taxon}$	-1259.26	0.67	0.68 [0.34:0.8]	0.13 [0.06:0.54]	0.85 [0.75:0.93]
7. $\beta_0 + \beta_1 \times \log_{10}(\text{mass})$ + $\beta_2 \times \text{taxon} + \beta_3 \times$ $\log_{10}(\text{mass}) \times \text{taxon}$	-1256.91	3.02	0.69 [0.37:0.81]	0.13 [0.06:0.52]	0.85 [0.75:0.93]
<u>Granulocytes</u>					
3. β_0	-1395.22	78.31	0.88 [0.8-0.92]		0.88 [0.8:0.92]
4. $\beta_0 + \beta_1 \times \text{taxon}$	-1395.46	78.07	0.81 [0.44:0.9]	0.03 [1.2e-4:0.49]	0.9 [0.82:0.96]
5. $\beta_0 + \beta_1 \times \log_{10}(\text{mass})$	-1465.85	7.68	0.71 [0.58:0.82]	0.12 [0.06:0.19]	0.83 [0.76:0.9]
6. $\beta_0 + \beta_1 \times \log_{10}(\text{mass})$ + $\beta_2 \times \text{taxon}$	-1466.3	7.23	0.71 [0.37:0.82]	0.1 [0.04:0.54]	0.85 [0.77:0.94]
7. $\beta_0 + \beta_1 \times \log_{10}(\text{mass})$ + $\beta_2 \times \text{taxon} + \beta_3 \times$ $\log_{10}(\text{Mass}) \times \text{taxon}$	-1473.53	0	0.7 [0.38:0.83]	0.11 [0.05:0.52]	0.87 [0.77:0.94]

Figure legends

Figure 1. Predictions based on flight influencing the scaling relationship between (left) lymphocyte and (right) granulocyte proportions and body mass. Animals in the figures represent the smallest and largest animals in the datasets. For the rationale of our predictions, please see the main text.

Figure 2. Posterior means and 95% credible intervals for coefficients of allometric scaling models applied to proportions of lymphocytes and granulocytes (neutrophils or heterophils) per each taxon and compared across taxa. Results are highlighted from the top models ($\Delta\text{DIC} < 5$, indicated by point size), whereas other competing models are transparent. Credible intervals that do not overlap with zero are displayed with solid lines. In the models comparing taxa, bats are represented by the intercept (models 4, 6, 7).

Figure 3. Observed scaling relationships in all species (bats ($n=60$), birds ($n=414$), and non-flying mammals ($n=256$)) between body mass and (A-C) lymphocyte and (D-F) neutr-/heterophil proportions. Dotted lines depict 95% credible intervals of the slope estimates. Data are plotted from model 2 (mass inclusive) from exercise 1.

Figure 1.

Figure 2.

Figure 3.

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