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-volant 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 comparative framework to quantify scaling relationships between body mass and the proportions of two types of white blood cells--lymphocytes, and granulocytes (neutr-/heterophils)--across 63 bat species, 400 bird species, and 251 non-volant mammal species. By using phylogenetically-informed statistical models on field-collected data from wild Neotropical bats and from captive bats, 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. Our inability to distinguish bat lymphocyte scaling from birds and bat granulocyte scaling from all other taxa suggest there may be other ecological explanations (i.e. not flight-related) for the cell proportion scaling patterns. 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 and its role in tolerance of (zoonotic) infections.

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 to 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-volant 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). In general, larger animals traverse greater distances with each step, have larger home ranges, and have larger respiratory and digestive tract surface area, meaning that they are likely at greater risk of pathogen exposure over their long lifespans (Calder 1984; Dobson and Hudson 1986; Han et al. 2015; Ruhs et al. 2020). These factors 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; Wiegel 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. Importantly, patterns for cell concentrations (and other aspects of immunity like antimicrobial capacity) do not follow the hypoallometric pattern that we would

expect based on metabolic rate (i.e. at a rate of -0.25) and instead support the Safety Factor Hypothesis. The Safety Factor Hypothesis proposes that larger animals favor infection risk reduction by investing heavily in baseline safety (Downs et al., 2020; Harrison, 2017), namely by using a reserve pool of broadly protective granulocytes (e.g., neutrophils and heterophils). However, concentrations of granulocytes in birds scale at a steeper rate (b=0.19; Ruhs et al., 2020) than in 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 pathogen 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). As reaching a large body size generally involves a long maturation time, this puts larger animals at increased risk of exposure or infection with pathogens over their long lifespans (Tian et al. 2015; Harrison 2017). 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 pathogens without education or much specificity (Lanier, 2013), although high concentrations of these cells can also promote tissue damage (Smith 1994). 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; Becker et al. 2020a). Bats were captured using mist nets (monitored continuously from approximately 19:00 to 22:00) and harp traps (monitored every half-hour from 18:00 to 22:00 and then at 5:00 the following morning); all individuals 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). Most bats were sampled within 1-3 hours of capture, and 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 (AMNHIACUC- 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). In our dataset, our smallest bat species is the Proboscis bat (*Rhynchonycteris naso*) at ~3.8g and our largest bat species the large flying fox (*Pteropus vampyrus*) at 935.9g. Within-species sample size ranged from 1 to 160 ($\bar{x}$=21 ± 4) but did not predict proportions of either cell type (neutrophils: ρ=-0.11, p=0.41; lymphocytes: ρ=0.09, p=0.48). We then extracted species means of lymphocyte and neutrophil proportions in whole blood from ZIMS (Species 360, 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. As bats are rare in captivity, the ZIMS dataset only included nine species, six of which were species we had in our dataset from the literature. Therefore we calculated species averages for each cell type and the final bat dataset include 63 species.

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). We removed bat data (n=3) from the extracted ZIMS mammal database and any non-volant birds from the ZIMS bird database (n=14). 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 (Tacutu et al., 2013), the Animal Diversity Website (Jones et al., 1997), and the Encyclopedia of Life (Parr et al., 2014). In our bird dataset, our body mass ranged from a 13.8g Gouldian finch (*Erythrura gouldiae*) to a 22600g Andean condor (*Vultur gryphus*). In our non-volant mammal dataset, our body mass ranged from a ~124g Pygmy marmoset (*Callithrix pygmaea*) to a 3824540g African elephant (*Loxodonta africana*).

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 (ΔDIC) to identify the best-fit GLMM. We defined the top model as that with the lowest DIC, and we considered models within ΔDIC<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 63 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=400) and non-volant mammals (n=251) independently to facilitate direct comparisons with bats. Results from bird and non-volant 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:

Model 1. $\log_{10}(\text{leukocyte proportion}) = \log10(a) + \varepsilon$

Model 2. $\log_{10}(\text{leukocyte proportion}) = \log10(a) + b * \log10(\text{body mass}) + \varepsilon$

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=63), birds (n=400), and non-volant mammals (n=251). Specifically, we fit five models to the data and compared DIC scores to determine the best-fit versions

Model 3. $\log_{10}(\text{leukocyte proportion}) = \log10(a) + \varepsilon$

Model 4. $\log_{10}(\text{leukocyte proportion}) = \log10(a) + b * \text{taxon} + \varepsilon$

Model 5. $\log_{10}(\text{leukocyte proportion}) = \log10(a) + b * \log10(\text{body mass}) + \varepsilon$

Model 6. $\log_{10}(\text{leukocyte proportion}) = \log10(a) + b_1 * \text{taxon} + b_2 * \log10(\text{body mass}) + \varepsilon$

Model 7. $\log_{10}(\text{leukocyte proportion}) = \log10(a) + b_1 * \text{taxon} + b_2 * \log10(\text{body mass}) + b_3 * \log10(\text{body mass}) * \text{taxon} + \varepsilon$

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=-0.06, -0.21:0.09) and neutrophils (*b*, CI=0.06, -0.1:0.2) were indistinguishable from zero (Table 2, 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 34% of the variation and body mass accounted for <1% of the variation. For neutrophils, again, phylogeny accounted for 18% of the variation and body accounted for < 1% of the variation. For both leukocyte types, the model fit of the mass model was 38-40% and did not increase much from the 27-33% 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 S1) for both cell types; lymphocytes scaled hypometrically (*b*, CI=-0.07, -0.09:-0.06; Table S2; Fig. 3) and heterophils scaled hypermetrically (*b*, CI=0.08, 0.07:0.1; Table S2). For non-volant mammals, the lymphocyte and neutrophil mass models were also the best-supported (Table S1); lymphocytes scaled hypometrically (*b*, CI=-0.08, -0.1:-0.06; Table S2; Fig. 3) and neutrophils scaled hypermetrically (*b*, CI=0.04, 0.02:0.05; Table S2). Phylogeny explained between 61-63% of the variation in birds and 57-69% of the variation in non-volant mammals (Tables S2). Bird and non-volant mammal granulocytes and non-volant 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 3, Fig. 2), the model with independent effects of mass and taxon (model 6; ΔDIC=0.3), and the model in which allometries differed between taxa (model 7; ΔDIC=4.85). For the mass model (model 5), phylogeny explained a large proportion of the variance (~74%), but the addition of mass increased explanatory power by 12% (marginal R^2). For model 6, the addition of mass and taxon increased explanatory power by an additional 11% (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 S3). Because the slope for bat lymphocyte proportions and body mass was indistinguishable from zero (from model 2, *b*, CI=-0.06, -0.21:0.09; exercise 1), this effect was largely driven by differences in the intercept between taxa (-0.32 in bats; -0.17 in birds; -0.08 in non-volant mammals). In other words, mean lymphocyte proportions across all body sizes were lower in bats than other mammals.

Granulocyte proportions were best explained by the model in which intercepts of allometries varied among taxa (model 7); this was driven by a universal hypermetric scaling slope across all species (*b*, CI= 0.06, 0.004:0.11). Phylogeny explained 57% of the total variation. Inclusion of mass and taxa increased explanatory power by 13% (marginal R^2). However, there was equal support for model 5 (ΔDIC=3.29) and model 6 (ΔDIC=2.37). For the mass model (model 5), phylogeny explained a large proportion of the variance (~78%), but the addition of mass increased explanatory power by 12% (marginal R^2). For model 6, the addition of mass and taxon increased explanatory power by an additional 11% (marginal R^2). 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 S4). 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 the architecture of their immune system (McGuire and Guglielmo, 2009). Here, we quantified the scaling relationships for proportions of two leukocyte types across 63 species of bats and compared these patterns across other vertebrate endotherms. Broad, comparative analyses of immunity between bats and other taxa are generally rare (Becker et al., 2019; Shaw et al., 2017), and allometry provides a powerful analytic framework for systematically comparing such data across species (Downs et al., 2020, Ruhs et al., 2020). Therefore, our analyses aimed to shed light on variation in 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 with 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 of bat immune systems having some distinctions from other endotherms but also suggest that physiological alterations to facilitate flight may not explain the allometry of cell proportions. Further, because birds display a much steeper hypermetric slope for granulocytes (Ruhs et al. 2020), avian scaling patterns are likely driven by other physiological, life-history, or exposure risk factors, rather than flight, that facilitated the need for larger birds to have disproportionately more circulating cells. We caution that our inability to distinguish bats from other endotherms in exercise 2 is likely driven by the high variation in bat data, thereby complicating identifying allometric patterns among taxa. Additionally, and from an applied perspective, allometry of immune cells may also provide limited inference into differences in traits such as pathogen tolerance compared to other, more specific immune measures like antimicrobial capacity and immune-associated gene expression. For leukocytes in particular, having more granulocytes may even promote pathology rather than host protection (Smith 1994). However, comparative analyses such as these can still provide insights into how flight has shaped general immune

scaling patterns and help generate predictions for future research into more functional immunological differences among taxa. Below, we focus on the results from exercise 1 to discuss taxon-specific scaling patterns 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?

As reaching a large body size requires a slow pace of life and involves greater resource turnover, larger animals are likely at greater risk of pathogen exposure (Lee 2006; Tian et al. 2015; Harrison 2017). Due to their long lifespans, increased energetic demands, and adaptive constraints associated with flight, large fliers likely have greater risk of pathogen exposure, even more so than large mammals. Thus, we predicted that bats, like birds (Ruhs et al. 2020), might need a disproportionately greater proportion of broadly protective cells 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 for allometric scaling in bat cell proportions. 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 most 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; Johnstone et al. 2012; Herrera et al., 2019). For example, while captive animals are generally thought to have many constraints alleviated (e.g. *ad libitum* food, less pathogen exposure), which would lessen the need for investment in cellular defense, stress of captivity and handling can cause a decrease in lymphocytes and an increase in heterophils

(Tian et al. 2015; Parker Fischer and Romero 2019). Further, physiological adjustment to captivity and handling is likely species-dependent and could take months (Parker Fischer and Romero 2019). The vertebrate response to stress can alter white blood cell counts within minutes to hours, depending on the species and even across taxa and possibly with body size (Davis et al. 2008; Johnstone et al. 2012). In our wild-derived bat data from Belize and the broader literature, many blood samples were collected within two hours from capture, although some studies did not include these details or had longer gaps between capture and processing. Importantly, exclusion of these studies or individuals known to be held for long durations did not influence the lack of scaling patterns of bat leukocytes (bat-only model 2; lymphocytes *b*, CI: -0.09, -0.23-0.04; neutrophils *b*, CI: 0.07, -0.06-0.23).

The absence of allometry in our bat data (when analyzed alone in exercise 1), which are primarily 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 (Martin et al., *in review*). Further, mean heterophil proportions were lower in wild versus captive birds. Similarly, comparison of wild and captive rodents also revealed no differences between scaling patterns of total leukocytes or neutrophil counts, despite wild animals having lower mean lymphocyte counts than captive 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; however, data from wild populations are likely ideal to assess the drivers and consequences of variation in immunity (Tian et al. 2015; Becker et a. 2020b). Lastly, the relatively smaller sample size of bats (n=63) compared to birds and non-volant mammals (n=400 and 251, respectively) is also unlikely to drive the greater variance in our bat data. Randomly subsampling our bird and non-volant mammal data produced approximately equivalent estimates of intercepts (bird lymphocytes=-0.09(-0.45:0.21), heterophils=-0.6(-0.95:-0.28); non-volant mammal lymphocytes=-0.08(-0.41:0.2), neutrophils=-0.43(-0.63:-0.24) and slopes (bird lymphocytes=-0.11(-0.14:-0.07), heterophils=0.1(0.06:0.13); non-volant mammal lymphocytes=-0.09(-0.12:-0.04), neutrophils=0.03(0.01:0.06)) for model 2, suggesting that the lack of allometry in bats is also unlikely driven by sample size.

The small differences in bat versus other taxa leukocyte 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). The potential lack of cell allometry in bats suggests yet another distinct aspect of immunology in these flying mammals. However, our inability to allometrically distinguish bats from birds (for lymphocytes) and bats from all other taxa (for neutrophils) in our cross-taxa comparison also suggest there may be other ecological explanations rather than flight itself for the observed leukocyte proportion scaling patterns.

Although it is most likely that our results from exercise 1 support a lack of allometry in bats (as described above), it is impossible to entirely discount the alternative explanation for our results in that the bat data could instead represent isometry. Isometry would mean that bat species require the same proportions of leukocytes across body size, which could be driven by certain distinct aspects of bat biology such as their slow life-history. Bats, even more so than birds, are long-lived such that selection for safety and disease risk-reduction is likely uniformly prioritized to accommodate longevity across body mass. 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, the 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 generally equal across body size. To reiterate, 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 instead represent isometry of lymphocyte proportions 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 hosts (Brook and Dobson, 2015; Halpin et al., 2011; Li et al., 2005; Luis et al., 2013). Investigating allometric scaling patterns of immunological features, including but not limited to 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 the difficulty in identifying allometric patterns of cell proportions (i.e., which are bound between 0% and 100%) compared to the previous discovery of hypermetric scaling of cell concentrations (Downs et al., 2020; Ruhs et al., 2020). Future studies interested in immune allometry instead should measure cell concentrations, which are not bound by proportional limits and provide greater insight into the total stock of cellular immunological resources. 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 immunological patterns, we encourage greater quantification of immune components across the bat phylogeny, specifically within 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. Such work could further differentiate evolutionary effects from those of flight and other aspects of life history on immune defense and provide insights into the traits that promote pathogen tolerance.

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Data accessibility: Bat leukocyte data and endotherm metadata are available as a supplemental file. There are three species of bats where leukocyte data are missing and that is because they originate from Species 360. All captive bird and mammal leukocyte data are available from Species360.

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Figures

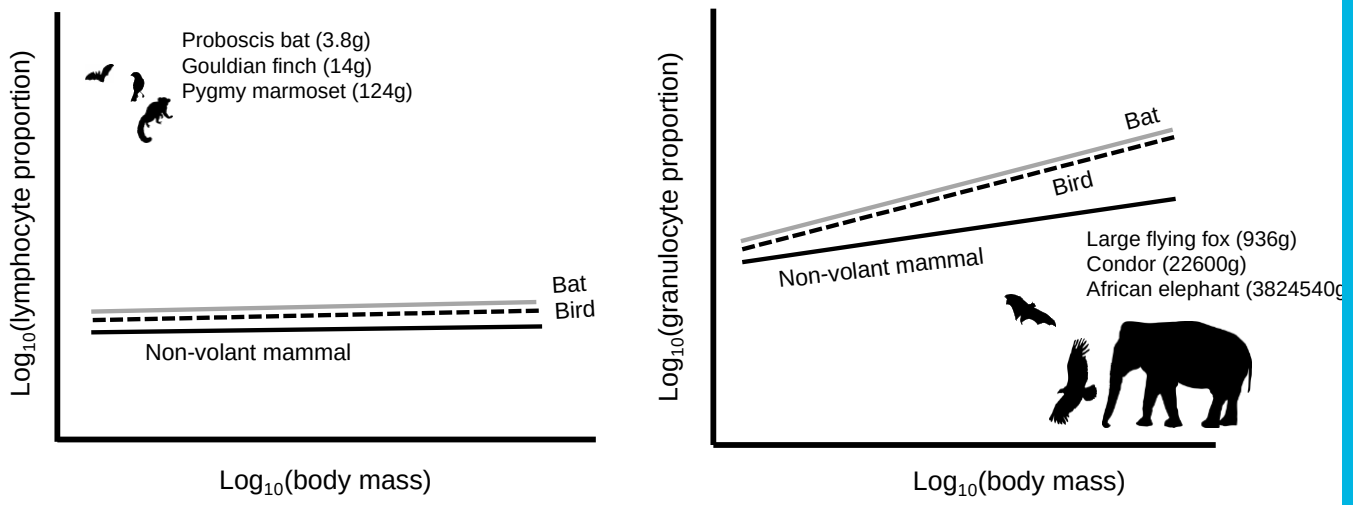

Figure 1. Predictions based on flight influencing the scaling relationship between (left) lymphocyte and (right) granulocyte proportions and body mass. Animal silhouettes in the figures represent the smallest and largest animals in the datasets. For the rationale of our predictions, please see the main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

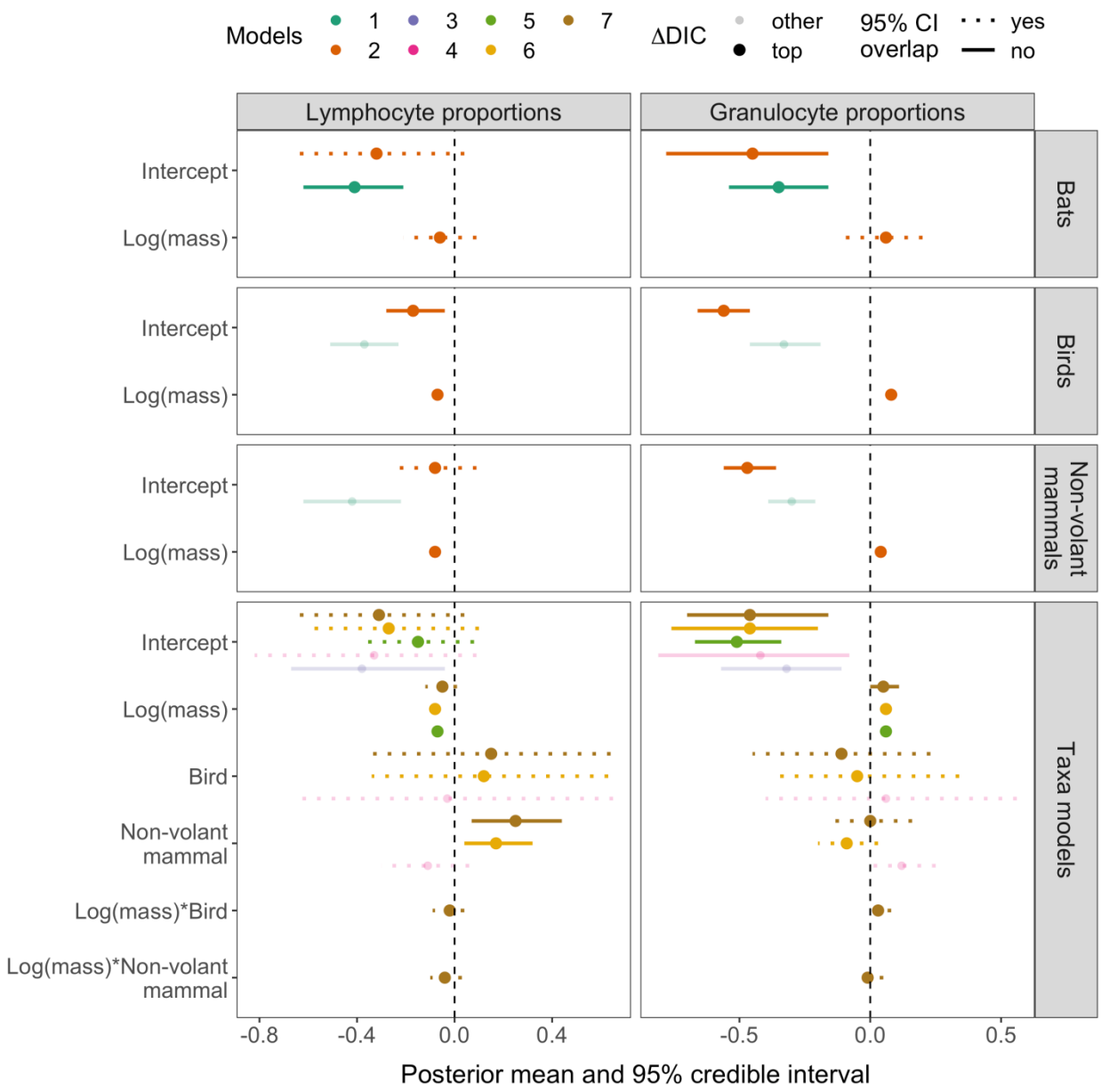

Figure 2. Posterior means and 95% credible intervals for coefficients of allometric scaling models applied to proportions of lymphocytes and granulocytes (neutrophils or heterophils) per each taxon and compared across taxa. Results are highlighted from the top models (ΔDIC<5, indicated by point size), 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).

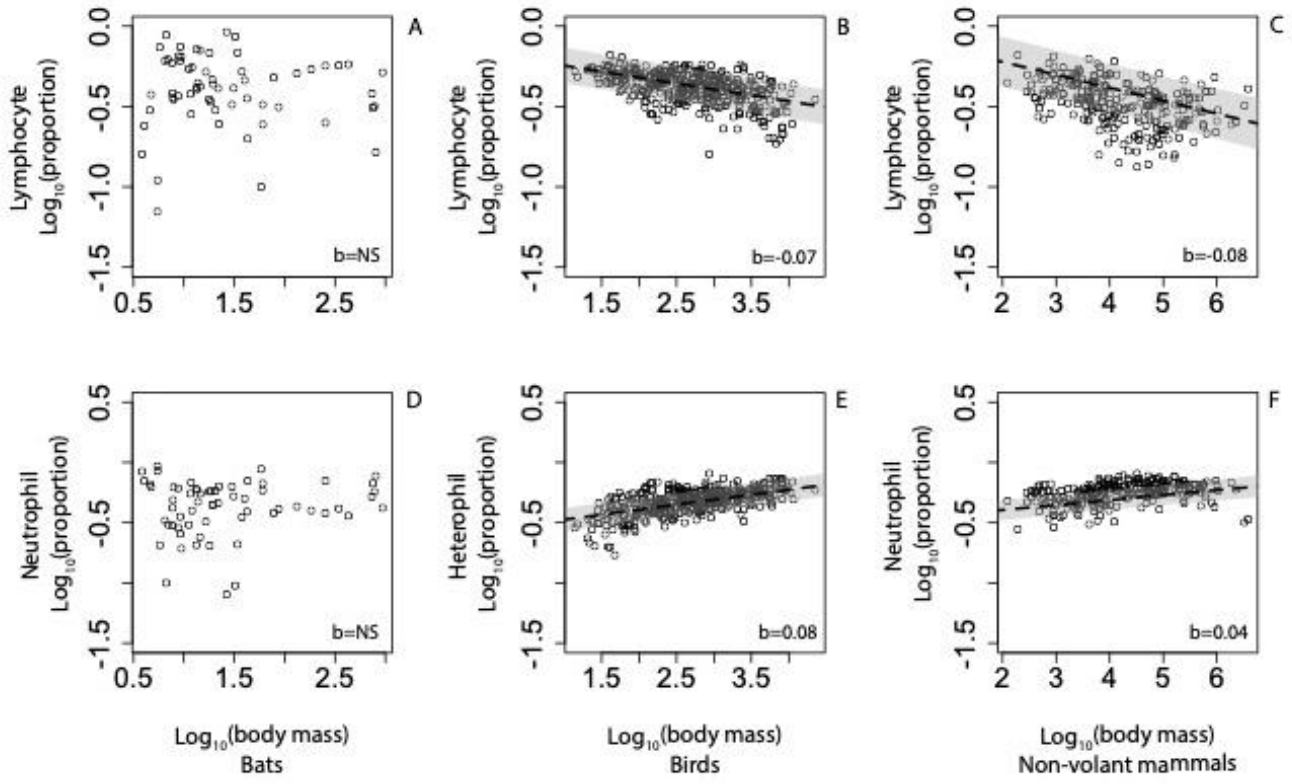

Figure 3. Observed scaling relationships in all species (bats (*n*=63), birds (*n*=400), and non-volant mammals (*n*=251)) between body mass and (A-C) lymphocyte and (D-F) neutr-/heterophil proportions. Dotted lines depict 95% credible intervals of the slope estimates. Data are plotted from model 2 (mass inclusive) from exercise 1.

Table 1. Best-fit models predicting circulating leukocyte concentrations in 63species of bats (exercise 1). Models test for the effects of body mass on $\log_{10}$-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 R^2 values to determine how much variation in leukocyte concentrations was explained by fixed effects and (3) conditional R^2 for overall model fit.

Model	**DIC**	**ΔDIC**	**λ (unadjusted) [95% CI]**	**Marginal R^2 [95% CI]**	**Conditional R^2 [95% CI]**
Lymphocytes					
1. β_0	-10.69	0.0	0.33 [0.06:0.73]		0.33 [0.06:0.73]
2. $\beta_0 + \beta_1$ x $\log_{10}$(Mass)	-10.57	0.12	0.34 [0.09:0.76]	$8.5e^{-4}$ [$2.13e^{-8}$:0.15]	0.4 [0.1:0.8]
Neutrophils					
1. β_0	-10.44	0.0	0.27 [0.06:0.66]		0.27 [0.06:0.66]
2. $\beta_0 + \beta_1$ x $\log_{10}$(Mass)	-9.92	0.52	0.18 [0.06:0.65]	0.001 [1.23e-7:0.17]	0.38 [0.08:0.7]

Table 2. Slope coefficients (b) and credible intervals (CI) of fixed effects in the mass model (model 2) for lymphocyte and neutrophil concentrations among 63 species of bats. There was equal support (ΔDIC<5) for model 1 (intercept-only) and model 2. Posterior mean is the mean of the posterior distribution.

Model		**Posterior mean**	**l-95% CI**	**u-95% CI**
Lymphocytes				
2. $\beta_0 + \beta_1$ x $\log_{10}$(Mass)	Intercept	-0.32	-0.64	0.04
	Log(mass)	-0.06	-0.21	0.09
Neutrophils				
2. $\beta_0 + \beta_1$ x $\log_{10}$(Mass)	Intercept	-0.45	-0.78	-0.16
	Log(mass)	0.06	-0.1	0.2

Table 3. 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	ΔDIC	λ (unadjusted) [95% CI]	Marginal R^2 [95% CI]	Conditional R^2 [95% CI]
Lymphocytes					
3. β_0	-1197.43	71.55	0.9 [0.83:0.94]		0.9 [0.83:0.94]
4. $\beta_0 + \beta_1$ x taxon	-1197.6	71.38	0.84 [0.41:0.92]	0.2 [0.0002:0.53]	0.92 [0.84:0.97]
5. $\beta_0 + \beta_1$ x log_{10}(mass)	**-1268.98**	**0.0**	**0.74 [0.61:0.84]**	**0.12 [0.07:0.19]**	**0.85 [0.78:0.92]**
6. $\beta_0 + \beta_1$ x log_{10}(mass) + β2 x taxon	**-1268.68**	**0.3**	**0.71 [0.36:0.85]**	**0.11 [0.04:0.55]**	**0.89 [0.77:0.94]**
7. $\beta_0 + \beta_1$ x log_{10}(mass) + β2 x taxon + β3 x log_{10}(mass)*taxon	**-1264.13**	**4.85**	**0.71 [0.36:0.83]**	**0.13 [0.05:0.54]**	**0.86 [0.75:0.94]**
Granulocytes					
3. β_0	-1347.45	52.98	0.86 [0.78:0.92]		0.86 [0.78:0.92]
4. $\beta_0 + \beta_1$ x taxon	-1346.46	53.97	0.82 [0.4:0.89]	0.03 [0.003:0.51]	0.86 [0.79:0.95]
5. $\beta_0 + \beta_1$ x log_{10}(mass)	**-1397.14**	**3.29**	**0.68 [0.53:0.79]**	**0.12 [0.07:0.19]**	**0.79 [0.7:0.87]**
6. $\beta_0 + \beta_1$ x log_{10}(mass) + β2 x taxon	**-1398.06**	**2.37**	**0.63 [0.37:0.8]**	**0.11 [0.05:0.49]**	**0.82 [0.7:0.91]**
7. $\beta_0 + \beta_1$ x log_{10}(mass) + β2 x taxon + β3 x log_{10}(Mass)*taxon	**-1400.43**	**0.0**	**0.57 [0.34:0.76]**	**0.13 [0.06:0.52]**	**0.79 [0.68:0.92]**

Table S1. Best-fit models predicting circulating leukocyte concentrations in 400 species of birds and 251 species of mammals. Models test for the effects of body mass on $\log_{10}$-transformed lymphocyte and granulocyte concentrations.

Taxon	Model	DIC	ΔDIC	λ (unadjusted) [95% CI]	Marginal R^2 [95% CI]	Conditional R^2 [95% CI]
Birds	**Lymphocytes**					
	1. β_0	-1001.64	53.34	0.89 [0.76:0.93]		0.89 [0.76:0.93]
	2. $\beta_0 + \beta_1$ x $\log_{10}$(Mass)	-1054.98	0.0	0.63 [0.49:0.79]	0.15 [0.07:0.22]	0.79 [0.69:0.88]
	Heterophils					
	1. β_0	-1018.19	81.09	0.84 [0.75:0.91]		0.84 [0.75:0.91]
	2. $\beta_0 + \beta_1$ x $\log_{10}$(Mass)	-1099.28	0.0	0.61 [0.44:0.73]	0.2 [0.11:0.27]	0.77 [0.7:0.86]
Non-volant mammals	**Lymphocytes**					
	1. β_0	-434.72	36.79	0.9 [0.76:0.95]		0.9 [0.76:0.95]
	2. $\beta_0 + \beta_1$ x $\log_{10}$(Mass)	-471.51	0.0	0.69 [0.51:0.85]	0.12 [0.05:0.23]	0.86 [0.72:0.92]
	Neutrophils					
	1. β_0	-602.76	20.36	0.74 [0.5:0.88]		0.74 [0.5:0.88]
	2. $\beta_0 + \beta_1$ x $\log_{10}$(Mass)	-623.12	0.0	0.57 [0.39:0.78]	0.09 [0.03:0.18]	0.7 [0.053:0.84]

Table S2. Slope coefficients (b) and credible intervals (CI) of fixed effects in the mass model (model 2) for lymphocyte and granulocyte concentrations among 400 species of birds and 251 species of mammals. For birds, model 2 outcompeted (>53 ΔDIC) model 1 (intercept-only). Posterior mean is the mean of the posterior distribution. For non-volant mammals, model 2 outcompeted (>20 ΔDIC) model 1 (intercept-only). Posterior mean is the mean of the posterior distribution.

Taxon	Model		Posterior mean	l-95% CI	u-95% CI
Birds	**Lymphocytes**				
	2. $\beta_0 + \beta_1$ x $\log_{10}$(mass)	Intercept	-0.17	-0.28	-0.04
		Log(mass)	-0.07	-0.09	-0.06
	Heterophils				
	2. $\beta_0 + \beta_1$ x $\log_{10}$(mass)	Intercept	-0.56	-0.66	-0.46
		Log(mass)	0.08	0.07	0.1
Non-volant mammals	**Lymphocytes**				
	2. $\beta_0 + \beta_1$ x $\log_{10}$(mass)	Intercept	-0.08	-0.25	0.09
		Log(mass)	-0.08	-0.1	-0.06
	Neutrophils				
	2. $\beta_0 + \beta_1$ x $\log_{10}$(mass)	Intercept	-0.47	-0.56	-0.36
		Log(mass)	0.04	0.02	0.05

Table S3. Intercepts, slope coefficients (*b*; posterior mean) and credible intervals (CI) of fixed effects in the direct bat/bird/non-volant mammal analysis for **lymphocyte** concentrations among 63 species of bats, 400 species of birds and 251 species of non-volant mammals. Posterior mean is the mean of the posterior distribution. Top models are denoted with bolded text.

Model		**Posterior mean**	**l-95% CI**	**u-95% CI**
3. Null	Intercept	-0.38	-0.67	-0.04
4. Taxon	Intercept	-0.33	-0.82	0.09
	Taxon – Bird	-0.03	-0.64	0.65
	Taxon – Mammal	-0.11	-0.3	0.06
5. Mass	Intercept	-0.15	-0.37	0.08
	Log(mass)	-0.07	-0.09	-0.06
6. Mass+ Taxon	Intercept	-0.27	-0.61	0.1
	Log(mass)	-0.08	-0.1	-0.07
	Taxon – Bird	0.12	-0.34	0.63
	Taxon – Mammal	0.17	0.04	0.32
7. Mass + Taxon + Mass*Taxon	Intercept	-0.31	-0.67	0.04
	Log(mass)	-0.05	-0.12	0.01
	Taxon – Bird	0.15	-0.34	0.64
	Taxon – Mammal	0.25	0.07	0.44
	Log(mass)*Taxon – Bird	-0.02	-0.09	0.04
	Log(mass)*Taxon - Mammal	-0.04	-0.1	0.03

Table S4. Intercepts, slope coefficients (*b*; posterior mean), and credible intervals (CI) of fixed effects in the direct bat/bird/mammal analysis for **granulocyte** concentrations among 63 species of bats, 414 species of birds and 256 species of non-volant mammals. Posterior mean is the mean of the posterior distribution. Top models are denoted with bolded text.

Model		**Posterior mean**	**l-95% CI**	**u-95% CI**
3. Null	Intercept	-0.32	-0.57	-0.11
4. Taxon	Intercept	-0.42	-0.81	-0.08
	Taxon – Bird	0.06	-0.4	0.56
	Taxon – Mammal	0.12	-0.02	0.25
5. Mass	Intercept	-0.51	-0.67	-0.34
	Log(mass)	0.06	0.05	0.07
6. Mass+ Taxon	Intercept	-0.46	-0.76	-0.2
	Log(mass)	0.06	0.05	0.07
	Taxon – Bird	-0.05	-0.37	0.34
	Taxon – Mammal	-0.09	-0.2	0.03
7. Mass + Taxon + Mass*Taxon	Intercept	-0.46	-0.7	-0.16
	Log(mass)	0.05	0.003	0.11
	Taxon – Bird	-0.11	-0.45	0.23
	Taxon – Mammal	-0.0002	-0.17	0.16
	Log(mass)*Taxon – Bird	0.03	-0.03	0.08
	Log(mass)*Taxon - Mammal	-0.01	-0.06	0.05

Table S5

Click here to download Table S5

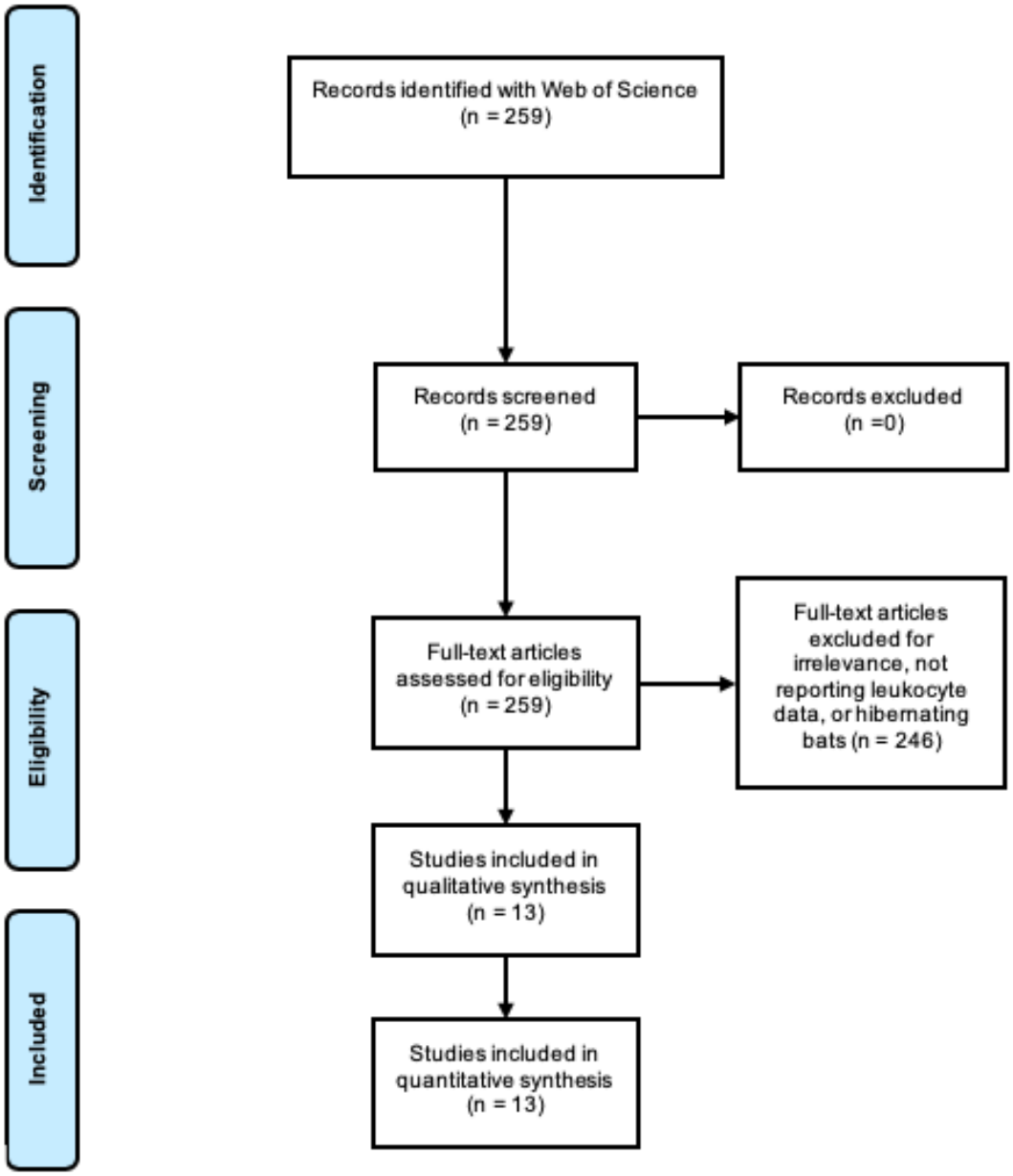

Figure S1. The data collection and inclusion process for studies of bat leukocyte proportions (PRISMA diagram). Searches used the following string: TS=(bat OR Chiroptera OR flying fox) AND (hematology OR white blood cell OR leukocyte). Searches were run in May 2020. Publications were excluded if they did not assess differential white blood cell counts in bats.

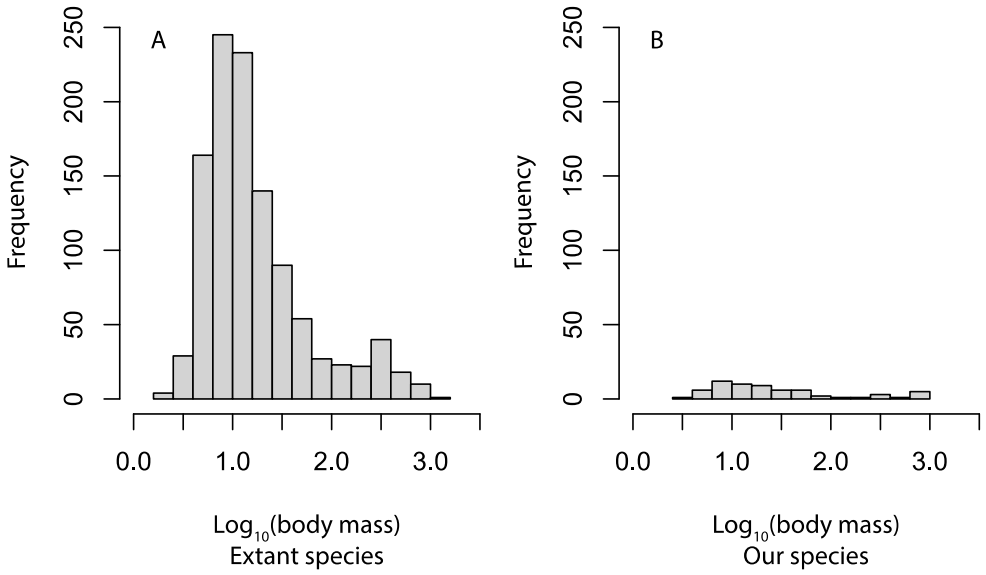

Figure S2. The frequency distribution of the (A) world's extant bat body mass (*n*=1100) and (B) the bat species used in this study (*n*=63). Data for panel A was taken from Elton traits (Wilman et al. 2014).