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The impacts of body mass on immune cell concentrations in birds

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Body mass affects many biological traits, but its impacts on immune defences are fairly unknown. Recent research on mammals found that neutrophil concentrations disproportionately increased (scaled hypermetrically) with body mass, a result not predicted by any existing theory. Although the scaling relationship for mammals might predict how leucocyte concentrations scale with body mass in other vertebrates, vertebrate classes are distinct in many ways that might affect their current and historic interactions with parasites and hence the evolution of their immune systems. Subsequently, here, we asked which existing scaling hypothesis best-predicts relationships between body mass and lymphocyte, eosinophil and heterophil concentrations—the avian functional equivalent of neutrophils—among more than 100 species of birds. We then examined the predictive power of body mass relative to life-history variation, as extensive literature indicates that the timing of key life events has influenced immune system variation among species. Finally, we ask whether avian scaling patterns differ from the patterns we observed in mammals. We found that an intercept-only model best explained lymphocyte and eosinophil concentrations among birds, indicating that the concentrations of these cell types were both independent of body mass. For heterophils, however, body mass explained 31% of the variation in concentrations among species, much more than life-history variation (4%). As with mammalian neutrophils, avian heterophils scaled hypermetrically ($b = 0.19 \pm 0.05$), but more steeply than mammals (approx. 1.5 ×; 0.11 ± 0.03). As such, we discuss why birds might require more broadly protective cells compared to mammals of the same body size. Overall, body mass appears to have strong influences on the architecture of immune systems.

1. Introduction

Body size influences almost all of the behaviours, physiological processes and hence eco-evolutionary roles of species in communities [1,2]. Nevertheless, despite the many life-history traits that we know to scale with body size and that might influence immune cell concentrations, we lack information on how body mass affects immune defences [3–5]. Many physiological factors that covary with body size evolution also probably impact exposure to parasites and pathogens and have implications for host immune systems [6,7]. A growing body of theory and limited data propose that body size influences the immune systems of species, partly because the risk of infection varies with body size [6–8]. In general, higher risk in large species may occur because large species cover greater physical distances with each movement (i.e. per step or beat of wings), have disproportionately larger home range sizes [9] meaning they come into contact with more habitat types and travel farther [8], and have larger respiratory, digestive and sexual tissue surface areas for infection relative to parasites [4,8]. Large species also have distinct ecological traits (e.g. social groups, home range size, feeding or foraging behaviour) [9–11], as large size requires either rapid growth in a short period or long development and subsequently delayed maturation [12]. Several studies have revealed that long-living (slow) species with low reproductive rates and long development times (e.g. ostrich,

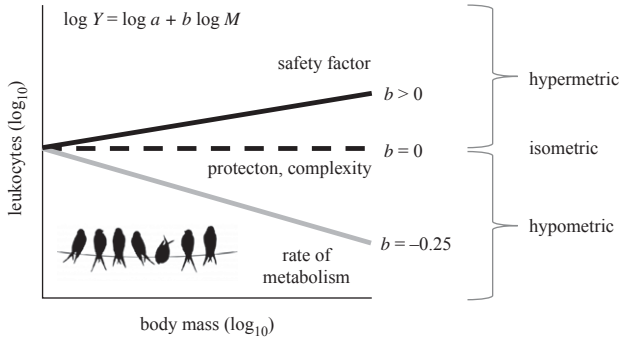

Figure 1. Possible scaling relationships between immune cell concentrations and body mass. Classic work has shown that many biological traits vary consistently with body size [9,17,18]. The equation in the figure represents the linearized function predicting the slope of the line relating body mass and leukocyte concentrations (i.e. *b*) for each predictive framework. For concentrations of immune cells, the safety factor hypothesis predicts a hypermetric relationship with body mass (black solid line; $b > 0$); the protecton/complexity hypothesis predicts isometric scaling or a mass-independent relationship (black dashed line; $b = 0$), and the rate of metabolism hypothesis predicts hypometric scaling (grey line; $b = -0.25$). Details of each hypothesis are described in the main text. Inset bird image from https://pixabay.com/illustrations/bird-birds-wire-perched-perching-220327/.

Struthio camelus, or elephant, *Elephas maximus*) have different immune defences than short-living (fast) ones (e.g. budgerigar, *Melopsittacus undulatus*, or mouse, *Mus musculus*) [13,14]. An individual should rely on specific immune defences (as opposed to generic defences) if they live for long periods because they may come into contact with the same pathogens repeatedly [15,16]. Yet to what degree these life-history patterns are genuine or just echoes of body mass effects remains unclear.

In the present study, we investigated whether and how body mass is related to immune cell concentrations among birds spanning several orders of magnitude in mass (approx. 1180-fold; electronic supplementary material, figure S1). Our particular aim was to identify which hypothesis best predicted the scaling relationships for immune cell concentrations among birds (figure 1) and then to test whether these scaling patterns are different from those found in mammals [19]. Scaling relationships are typically expressed by the equation $Y = aM^b$; however, log-transformation of this equation produces $\log(Y) = \log(a) + b \times \log(M)$, where a represents a constant, M body mass and Y the focal trait. The main parameter of interest, with regard to allometry, is b, which is the slope of the line relating to body masses and leucocyte concentrations. Traits that scale isometrically change in proportion to changes in body mass. For cell concentrations, the focal trait of this study, the isometric prediction is $b = 0$ or mass independence (black dashed line, figure 1) [9,17].

Two hypotheses predict isometric scaling for immune cell concentrations. The first is the protecton hypothesis, which proposes that all organisms require similar levels of protection, regardless of size [20,21]. The related complexity of the immune system hypothesis (henceforth the complexity hypothesis) [19] assumes that the time for surveillance of tissues and delivery of a single leucocyte is independent of body mass [22,23]. The rate of metabolism hypothesis [24] invokes metabolic rate as the key driver of variation in immune cell concentrations [1]. Thus, the rates of proliferation and actions of immune cells should be reliant on the mass-specific rate of metabolism of a host [24]. This hypothesis predicts a hypometric relationship between leucocyte concentration and body mass ($b = -0.25$, grey line, figure 1), such that large animals have disproportionately lower cell concentrations than small animals. A final hypothesis is derived from Downs *et al*.'s [19] recent discovery about neutrophil scaling in over 250 species of mammals and prior theory on the evolution of similar organismal functions [25]. This previous study found that neutrophil concentrations scaled hypermetrically with body mass ($b = 0.11$) [19]. In hypermetric scaling, traits of interest are disproportionately larger in large organisms than would be expected by geometry. For cell concentrations, hypermetric scaling occurs when $b > 0$ (black solid line, figure 1). The observed hypermetric scaling in neutrophils led to the safety factor hypothesis [19], which proposes that hypermetric scaling may have evolved for early anti-microbial defences, because larger species require disproportionately more fast-acting, broadly protective defensive cells to combat infections than small species [26]. In other words, we expect that this pattern evolved because large species must prioritize risk reduction over other life-history priorities as they tend to mature more slowly and hence live longer than smaller species [12,15,19].

Here, we sought to determine which hypothesis best-predicted scaling relationships for three leucocyte concentrations (heterophils, eosinophils and lymphocytes) among avian species and test whether any relationships compared to allometries in mammals [19]. To enable comparison with the mammalian work, we used the same analytical approach as Downs *et al*. [19]. We limited our analysis to captive zoo animals to attempt to control the inherent variation in wild populations. To our knowledge, there is only one other study that has investigated this relationship among birds; however, those data were gathered from both wild and captive species and the analysis was not phylogenetically controlled [27]. Therefore, there is a need to understand if phylogeny and other life-history characteristics contribute to allometric patterns. Subsequently, here, we conducted three modelling exercises. First, we asked which hypothesis best-predicted scaling relationships: the protecton/complexity hypothesis (isometric slope; figure 1), the rate of metabolism hypothesis (hypometric slope) or the safety factor hypothesis (hypermetric slope) [19]. For the safety factor hypothesis, we did not attempt to fit the same b as discovered in the mammalian study for heterophils and instead estimated the b from the data. We took this approach because birds and mammals differ in many ways that could influence the magnitude or direction of the slope. For instance, birds do not possess neutrophils, but instead maintain a functionally similar granulocyte called a heterophil. Neutrophils are derived from vascular spaces, which overall could provide mammals a potentially bigger cell pool (outside general circulation) on which to draw granulocytes [28]. Heterophils, by contrast, are derived from stem cells in the extravascular spaces of bone marrow or by extramedullary hematopoiesis. Heterophils are fast-acting leucocytes able to engulf or control microbes, typically by mechanisms involving oxidation, upon first exposure [29,30]. We thus expected similar hypermetric scaling as with mammals. Eosinophils also have diverse roles but are especially important in the control of helminth infections and other extracellular parasites [30]. Their functional resemblance to heterophils also led us to expect hypermetric scaling for this leucocyte type. Compared

to the other two cell types, lymphocytes are exceptionally heterogeneous [30]; an amalgam of quiescent and active B and T cells with diverse protective roles against intra- and extracellular macro- and microparasites through various mechanisms from the production of antibodies, to the coordination of other immune responses, to direct control of viral pathogens. For this leucocyte class, we expected the predictive power of body mass to be weak given the high functional diversity of this cell group. Most birds also lack the differentiated lymph nodes of mammals and instead maintain diffuse lymphoid tissue that creates local nodules wherever antigen-stimulation occurs [31]. Birds might therefore lack a tissue repository for some leucocytes [28], which would affect concentrations of leucocytes in circulation.

Our second modelling exercise focused on whether life-history traits (maximal lifespan, maximal reproductive capacity and the interaction of both terms with body mass) explained more variation in leucocyte concentrations in birds than body mass, after accounting for phylogeny. We expected body mass to explain more variation in all three cell types than either life-history variable, but that phylogeny would also be a strong predictor as observed previously [19,32–34]. We also expected the relative importance of body mass, life-history traits and phylogeny to vary among cell types because of the different defensive functions of each cell type. Our third and final modelling exercise directly tested whether the scaling patterns observed in birds and mammals for lymphocytes and heterophils/neutrophils differed from one another.

2. Materials and methods

(a) Trait data

We extracted species means of heterophil and lymphocyte concentrations (cells L^{-1}) in whole blood for 116 bird species and eosinophil concentrations for 88 bird species from the ZIMS database maintained by Species360 (means obtained from the number of samples collected, see sample size, electronic supplementary material, table S1) [35]. Cell concentration data came from captive, adult animals housed in zoos accredited by the Association of Zoos and Aquariums (AZA) and considered healthy [35]. We used only data from adult, captive individuals to minimize the confounding effects of age, infection and unknown reproductive status that would have arisen if we studied wild individuals. Zoo collections are biased toward large-bodied birds. Consequently, our analysis underrepresents small species (for a comparison to the world's bird species body mass distributions see electronic supplementary material, figure S1). We were conservative and used only global species reference intervals and absolute counts reported for each species (see Dryad for descriptions). We extracted data on body mass, maximum lifespan, age at maturation, inter-laying interval (i.e. how often a species lays a clutch), average clutch size and incubation period from the CRC Handbook of Avian Masses [36] and publicly available databases such as AnAge [37] and the Animal Diversity website [37]. Data were compiled as well as possible using a combination of all sources so as to have the most complete dataset. For one species, *Pteroglossus aracari*, age at maturation was estimated from a closely related species, *Pteroglossus viridis*. From these data, we calculated maximal reproductive capacity for each species as [19]

$$\text{maximal reproductive capacity} = \frac{\text{maximum lifespan} - \text{age at maturation}}{(\text{incubation period} + \text{inter} - \text{laying interval}) \times \text{clutchsize}}.$$

(b) Correlation analyses

To determine if cell concentrations were correlated, we conducted Pearson's correlation analyses between $\log_{10}$-transformed (i) lymphocytes and heterophils, (ii) lymphocytes and eosinophils, and (iii) heterophils and eosinophils. We also analysed correlations among body mass and life-history traits.

(i) Exercise 1: leukocyte allometry modelling

Our first interest was to determine which hypothesis best-predicted scaling relationships for three leucocyte concentrations among avian species. Therefore, we fitted three sets of *a priori* models to the $\log_{10}$-transformed concentrations of lymphocytes, heterophils and eosinophils, respectively (nine total models):

$$\text{model 1:}\quad \log_{10}(\text{leukocyte concentration}) = \log_{10}(a) + \varepsilon$$

$$\text{model 2:}\quad \log_{10}(\text{leukocyte concentration}) = \log_{10}(a) - \frac{1}{4} \times \log_{10}(\text{body mass}) + \varepsilon$$

$$\text{model 3:}\quad \log_{10}(\text{leukocyte concentration}) = \log_{10}(a) + b \times \log_{10}(\text{body mass}) + \varepsilon.$$

In these models, a represents the intercept, b the scaling coefficient and ε the error. model 1 was an intercept-only model that corresponded to the Protecton/Complexity Hypothesis (mass independence/isometry). model 1 was also the model we would expect based on a null allometric hypothesis that leucocyte concentrations are independent of body mass and it served as an intercept-only model for model comparisons. model 2 corresponded to the rate of metabolism hypothesis, which predicts hypometric scaling ($b = -0.25$). model 3 estimated the slope from the data which allowed us to test for hypermetric scaling ($b > 0$), predicted by the safety factor hypothesis.

(ii) Exercise 2: comparing effects of body mass relative to life-history

To compare the relative ability of life-history traits and body mass to explain concentrations of heterophils, eosinophils and lymphocytes, we fit two sets of *a priori* models for each cell type that included $\log_{10}$(body mass), maximum longevity, maximum reproductive capacity and all two-way interactions as fixed effects (six total models):

$$\text{life-history model:}\quad \log_{10}(\text{lymphocyte/heterophil/eosinophil concentration}) = \log_{10}(a) + \beta_1 \times \text{max reproductive capacity} + \beta_2 \times \text{max longevity} + \varepsilon.$$

$$\text{omnibus model:}\quad \log_{10}(\text{lymphocyte/heterophil/eosinophil concentration}) = \log_{10}(a) + \beta_1 \times \log_{10}(\text{body mass}) + \beta_2 \times \text{max reproductive capacity} + \beta_3 \times \text{max longevity} + \beta_4 \times (\log_{10}(\text{body mass}) \times \text{max reproductive capacity}) + \beta_5 \times (\log_{10}(\text{body mass}) \times \text{max longevity}) + \varepsilon.$$

Regardless of the top model from exercise 1, we compared the life-history and omnibus models to the model in which the scaling coefficient for mass was fitted by the data from exercise 1 (model 3). We used this approach to determine the amount of variation explained by life-history traits compared to body mass.

(c) Comparing models and determining fit

All analyses involved phylogenetic mixed-effects models in R (version 3.6.0) using the packages ape [38] and MCMCglmm [39]. For modelling exercises 1 and 2, we included phylogenetic effects from a tree we produced by pruning the time-rooted phylogenetic tree created by Uyeda *et al*. [40] to our species list, then

using that tree to create a phylogenetic covariance matrix. Our sample sizes differed among cell types ($n = 116$ for lymphocytes and heterophils and $n = 88$ for eosinophils) due to differences in the availability of data in Species360 and our life-history references. Therefore, we created and used two different phylogenetic trees, one for lymphocytes and heterophils and another for eosinophils (electronic supplementary material, figure S2). 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 except model 2, where we used *a priori* value of −0.25 [19]. Models were run for 260 k iterations with 60 k burn-in and a 200-iteration thinning interval.

First, we assessed model convergence by examining model trace and density plots. We then calculated model fits for all models from exercises 1 and 2 using conditional R^2 [41], and used the deviance information criterion (DIC) to identify the best-fit model. For exercise 1, we first compared models 1–3 and defined the top model as the model with the lowest DIC. If additional models had a $\Delta\text{DIC} < 5$, we considered these models to have equivalent support and included them within the top set of models [42]. For exercise 2, we compared the life-history and omnibus models to the mass-only model (model 3) in which the scaling coefficient for mass was fitted by the data from exercise 1. This approach enabled us to discern whether previous discoveries for life-history variable effects explained more variation than body mass alone. For all models, we also calculated (i) Pagel's unadjusted lambda to determine the variation explained by the phylogeny not accounting for fixed effects and (ii) marginal R^2 values to determine how much variation in leucocyte concentrations was explained by fixed effects [41,43].

(i) Exercise 3. direct comparison of bird and mammal slopes

Our third modelling exercise was designed to compare the slopes of the relationships between body mass and cell types among birds ($n = 116$) and mammals ($n = 259$). The mammal dataset included only lymphocytes and neutrophils, limiting our analyses to those cell types. We compared avian heterophils with mammalian neutrophils because they have similar functions [44]. We built a model similar to model 3 from exercise 1, except we also included the fixed effect term class (Aves or Mammalia) and the interaction between $\log_{10}$(body mass) and class. For the two models (lymphocytes and heter-/neutrophils, separately), we then calculated Pagel's unadjusted lambda, marginal R^2 values and conditional R^2 values [41,43].

$$\text{bird/mammal model: } \log_{10}(\text{lymphocyte/heterophil concentration}) = \log_{10}(a) + b_1 \times \log_{10}(\text{body mass}) + b_2 \times \text{class} + b_3 \times \log_{10}(\text{body mass}) \times \text{class} + \varepsilon.$$

We created a new phylogenetic covariance matrix estimated using a phylogenetic tree for both birds and mammals combined that was constructed using data from the National Center for Biotechnology Information and phyloT [45]. Polytomies were resolved during tree construction using the randomized function built into phyloT. The bird/mammal model was fitted with an inverse-gamma prior set to 0.01 and models run for 260 k iterations with 60 k burn-in and a 200-iteration thinning interval.

3. Results

(a) Correlation analyses

Log_{10}-transformed lymphocytes were not correlated with $\log_{10}$–transformed heterophils ($r = 0.102$, $t_{117} = 1.11$, $p = 0.269$), nor with $\log_{10}$-transformed eosinophils ($r = 0.164$, $t_{86} = 1.54$, $p = 0.127$). However, species with more $\log_{10}$-transformed heterophils had more $\log_{10}$-transformed eosinophils ($r = 0.402$, $t_{86} = 4.07$, $p = 0.0001$). We also found correlations between $\log_{10}$-transformed body mass and life-history traits (electronic supplementary material, figure S3).

(i) Exercise 1: best-fit models for leucocyte allometry

The Protecton/Complexity model (model 1) best-predicted avian lymphocyte and eosinophil concentrations, but the mass-only model (model 3) was within 2 ΔDIC of the intercept-only model for both leucocyte types (table 1) [42]. However, according to the marginal R^2 values, body mass was a weak predictor of variation in lymphocytes and eosinophils (model 3; electronic supplementary material, figure S4), explaining only less than 0.1% more variation in either lymphocyte or eosinophil concentrations (table 1). Overall, the conditional R^2 values indicated that, model 1 (null, intercept-only model) accounted for 58% of the variation in lymphocytes and 87% of the variation in eosinophils (table 1).

Outcomes differed for heterophil concentrations (table 1, figure 2*a*). First, when the slope coefficient was estimated from the data, the mass-only model (model 3) was best supported by the DIC values. The mass-only model (model 3) had an overall model fit of 83% and the marginal R^2 indicated that body mass alone accounting for 31% of the variation. In this model, heterophil concentrations scaled hypermetrically (model 3: b, 95% credible interval (CI) = 0.19, 0.14–0.24; figure 2*a*; electronic supplementary material, table S2). In all models for all leucocyte types, Pagel's unadjusted λ showed that appreciable phylogenetic effects were detected (unadjusted $\lambda > 43\%$; table 1).

(ii) Exercise 2: effects of life-history traits relative to body mass

The lymphocyte life-history and omnibus models were inferior to model 3 from exercise 1 (table 2; $\Delta\text{DIC} > 6.85$). The eosinophil omnibus model had equivalent support compared to model 3 from exercise 1 (table 2; $\Delta\text{DIC} = 1.22$). The heterophil omnibus model had more support than the mass-only model from exercise 1 (model 3; table 2) and the conditional R^2 value indicates that the omnibus model explained 83% of variation in heterophils. However, marginal R^2 value indicated that life-history traits, alone, explained only 4% of the total variation (table 2), compared to the mass-only model which explained 31% of the variation (marginal R^2; table 1). Interestingly, heterophils still scaled hypermetrically with body mass in the omnibus model (b, 95% CI = 0.21, 0.12 : 0.3; electronic supplementary material, table S2). In the light of these subtle effects of life-history variables on heterophil concentrations, we selected model 3 (mass-only; estimating b from the data) as the most parsimonious about the scaling of heterophils.

(iii) Exercise 3: direct comparison of bird and mammal slopes

The bird/mammal model for lymphocytes accounted for 80% of the variation (conditional R^2; electronic supplementary material, table S3); however, the fixed effects, body mass and class, explained only 2% of the variation (marginal R^2; electronic supplementary material, table S3). For heter-/neutrophils, this model explained 92% of the overall variation (conditional R^2; electronic supplementary material, table S3) with body mass and class explaining 11% of the total variation (marginal R^2; electronic supplementary material, table S3). Heter-/neutrophils scaled hypermetrically with body mass (b, 95% CI = 0.19, 0.14–0.24; electronic supplementary material, table S4). The interaction between body mass

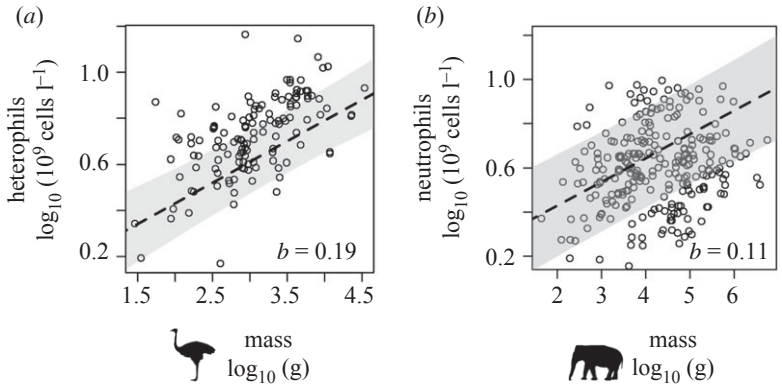

Figure 2. Observed scaling relationships between body mass and (*a*) heterophils from birds and (*b*) neutrophils from mammals. Shaded areas depict 95% credible intervals of the slope estimates in which only body mass (not life-history traits) are included in the model (avian model 3 here; the mammalian model is from [19]).

Table 1. The best-fit models from exercise 1 predicting circulating leukocyte concentrations in birds. Models with a ΔDIC < 5 were considered indistinguishable and within the top model set. Models test for the effects of body mass on $\log_{10}$-transformed lymphocyte, eosinophil and heterophil concentrations. Model 1 fitted a null model and corresponded with the predictions of the protection and immune complexity hypotheses, model 2 fit a slope of −0.25 to correspond with the predictions of the rate of metabolism hypothesis, and model 3 was allowed to estimate the slope given the data. model 3 allowed us to test for the hypermetric prediction of the safety factor hypothesis. For all models, we also calculated (i) Pagel's unadjusted lambda to determine the variation explained by the phylogeny controlling for fixed effects, (ii) marginal R^2 values to determine how much variation in leukocyte concentrations was explained by fixed effects and (iii) overall model R^2 values [44,45]. Here, *k* is defined as the number of parameters included in the model.

model	*k*	DIC	ΔDIC	λ (unadjusted) [95% CI]	marginal R^2 [95% CI]	model R^2
lymphocytes						
1. β_0	0	−219.39	0			0.58 [0.38–0.81]
3. $\beta_0 + \beta_1 x \log_{10}(\text{mass})$	1	−218.09	1.3	0.63 [0.4–0.83]	0.0002 [3.56×10^{-6} – 0.04]	0.66 [0.41–0.83]
2. $\beta_0 - \frac{1}{4} \times \log_{10}(\text{mass})$	1	−173.18	46.20	0.60 [0.49–0.74]	0.28 [0.21–0.36]	0.92 [0.83–0.96]
eosinophils						
1. β_0	0	−85.64	0			0.87 [0.76–0.98]
3. $\beta_0 + \beta_1 x \log_{10}(\text{mass})$	1	−84.89	0.75	0.89 [0.72–0.97]	0.0004 [1.06×10^{-6} – 0.09]	0.93 [0.78–0.98]
2. $\beta_0 - \frac{1}{4} \times \log_{10}(\text{mass})$	1	−83.53	2.12	0.87 [0.71–0.92]	0.1 [0.06–0.14]	0.95 [0.84–0.99]
heterophils						
3. $\beta_0 + \beta_1 x \log_{10}(\text{mass})$	1	−176.17	0	0.43 [0.23–0.72]	0.31 [0.15–0.43]	0.83 [0.61–0.91]
2. $\beta_0 - \frac{1}{4} \times \log_{10}(\text{mass})$	1	−151.28	24.89	0.85 [0.74–0.90]	0.12 [0.08–0.16]	0.96 [0.9–0.99]
1. β_0	0	−163.86	12.31			0.89 [0.64–0.95]

and class predicted cell concentrations (*b*, 95% CI = −0.1, −0.15: −0.04; electronic supplementary material, table S4) indicating that bird heterophils scaled more steeply (by $b = 0.09$) than mammal neutrophils. Taken together, these results suggest that the granulocyte allometry is more hypermetric in birds ($b = 0.19$) than mammals ($b = 0.11$) [16].

4. Discussion

Here, we investigated the scaling of three types of leucocytes across greater than 88 species of birds. Although our database underrepresented small birds due to biases in zoo collections, it did include data from 21 orders and an 1180-fold difference in body mass. We found no evidence for allometries for lymphocyte and eosinophil concentrations; the best-fit models for both cell types did not include body mass. These results are consistent with the protecton and complexity hypotheses, but we encourage caution in interpreting these results as support for it. Null models (model 1) provide weak evidence of isometry because it is unclear whether the slope is truly zero (isometry) or if there is an absence of a pattern. However, posterior distributions for the estimate of the scaling coefficient (*b*) for each leucocyte type centre near zero, which would be consistent with isometry.

Heterophils, by contrast, scaled hypermetrically regardless of the model used, although the omnibus model (i.e. with mass and life-history variables) performed better and explained the same amount of variation (83%) as the model with only body mass as a fixed effect (model 3). However,

Table 2. model results from exercise 2, comparing the effects of life-history metrics and body mass on $\log_{10}$-transformed lymphocyte, eosinophil and heterophil concentrations. We compared the results of each life-history and omnibus model to the mass-only model (model 3) and ΔDIC values presented here are in relation to model 3 results from table 1. [§]For heterophils, the ΔDIC was 4.72 lower than the ΔDIC of model 3 and therefore we infer a ΔDIC = 0.

model	*k*	DIC	ΔDIC	λ (unadjusted) [95% CI]	marginal R^2 [95% CI]	model R^2
lymphocytes						
life-history model[a]	2	−215.97	42.79	0.63 [0.33–0.78]	0.004 [7.7^{-5}–0.09]	0.56 [0.38–0.8]
omnibus model[b]	5	−212.54	6.85	0.51 [0.31–0.77]	0.04 [0.008–0.13]	0.54 [0.39–0.8]
eosinophils						
life-history model[a]	2	−77.73	7.91	0.87 [0.69–0.97]	0.002 [1.85^{-5}–0.07]	0.93 [0.73–0.99]
omnibus model[b]	5	−84.42	1.22	0.89 [0.64–0.94]	0.07 [0.01–0.18]	0.93 [0.78–0.98]
heterophils						
life-history model[a]	2	−154.76	21.41	0.75 [0.47–0.89]	0.04 [0.002–0.15]	0.81 [0.58–0.93]
omnibus model[b]	5	−180.89	0[§]	0.4 [0.19–0.61]	0.43 [0.28–0.55]	0.83 [0.64–0.92]

[a]$\beta_0 + \beta_1 \times$ max repro $+ \beta_2 \times$ max longevity.
[b]$\beta_0 + \beta_1 \times \log_{10}(\text{mass}) + \beta_2 \times$ max repro $+ \beta_3 \times$ max longevity $+ \beta_4 \times \log_{10}(\text{mass}) \times$ max repro $+ \beta_5 \times \log_{10}(\text{mass}) \times$ max longevity.

as body mass explained much more variation in heterophil concentrations (31%) than life-history traits (approx. 4%) and the combination of mass and life history (omnibus) only improved the explanatory capacity of the model by approximately 12% over the mass-only model, we favoured the mass-only model as our basis for subsequent analysis and interpretation. Our most striking discovery was the extreme hypermetric scaling of heterophil concentrations among birds (*b*, 95% CI = 0.19, 0.14 : 0.24). This coefficient supports the safety factor hypothesis and is much steeper than the estimate we described previously for mammals (*b*, 95% CI = 0.11, 0.09 : 0.14; figure 2*b*) [19]. While the correlative nature of our data cannot explain why avian heterophils scale more steeply than mammalian neutrophils, below we offer several possibilities warranting future investigation. First, however, we address the lack of scaling for the other two cell types.

(a) Lymphocyte and eosinophil scaling

Lymphocytes are a heterogeneous class of leucocytes, including both B and T cells. For example, in peripheral blood, 45.9% are T cells and 39.2% are B cells [46]. These cells have diverse responsibilities and can be in such different states of activation that it might be difficult to detect relationships between body mass and lymphocyte concentrations even if they exist. However, allometries have been detected for lymphocytes in several but not all other studies (all mammals ($b = -0.07$ [47]; −0.04 [19], primates ($b = -0.1$ [47,48]), carnivores [49], birds [27] and bats [50], but not rodents [51]). Avian and mammalian lymphocytes provide predominantly the same forms of protection [52], so it is interesting that such different patterns of scaling have been detected among species and studies. However, these previously reported scaling exponents might be misleading in the sense that many of them explain little of the variation and some do not account for the variance explained by phylogeny. For instance, in our previous mammalian study, body mass explained only 3% of variation among species in lymphocyte concentration [19] and the only other large scale comparison of birds did not use phylogenetically informed statistics [27]. It would be valuable to develop tools to characterize counts of classes of lymphocytes (e.g. CD4 versus CD8 T cells) that can be applied to phylogenetically broad species, which would enable us to study the scaling of functionally distinct lymphocyte classes.

The preceding logic for lymphocytes probably does not transfer to eosinophils. An absence of scaling for eosinophils is less likely derived from functional diversity among members of this class because all eosinophils perform the same type of defence. Eosinophils respond rapidly to local inflammation, mostly when it is induced by macroparasites [49,53]. In birds, we detected no effect of body mass on eosinophil concentrations, but this finding is inconsistent with work in bats [50], carnivores [48] and primates [48], all of which detected hypermetric scaling. The largest study to date in birds also found slight hypermetric scaling; however, those data were not log-transformed and statistics were not phylogenetically informed so direct comparisons to the current study are unadvised ($b = 0.048$) [27]. This discrepancy between our results and published results could be due to at least three factors. First, as with lymphocytes, body mass might have statistically significant, but subtle, influences on eosinophil concentrations in some taxa. Second, eosinophils are more common in tissues than in blood [53] (these data are from whole blood); therefore, scaling patterns might be more prominent if described from tissues. Third, and related to factor two, eosinophils generally circulate in low numbers in both birds and mammals [53,54], leaving simply too little variation to detect patterns across some clades. That is, a floor effect on total concentrations could impede the estimation of *b* for this cell type.

(b) Heterophil scaling

Our most compelling discovery involved the hypermetric *b* for heterophils. Heterophils are phagocytes that protect predominantly against microparasites. Much of our knowledge about avian heterophils comes from work on mammalian neutrophils, though, and most evidence indicates that these cell types share hematopoietic history and function [55]. Among mammal species and birds, heter-/neutrophil concentrations consistently scale hypermetrically (e.g. bats [50], rodents [51], carnivores [49], primates [47], mammals broadly

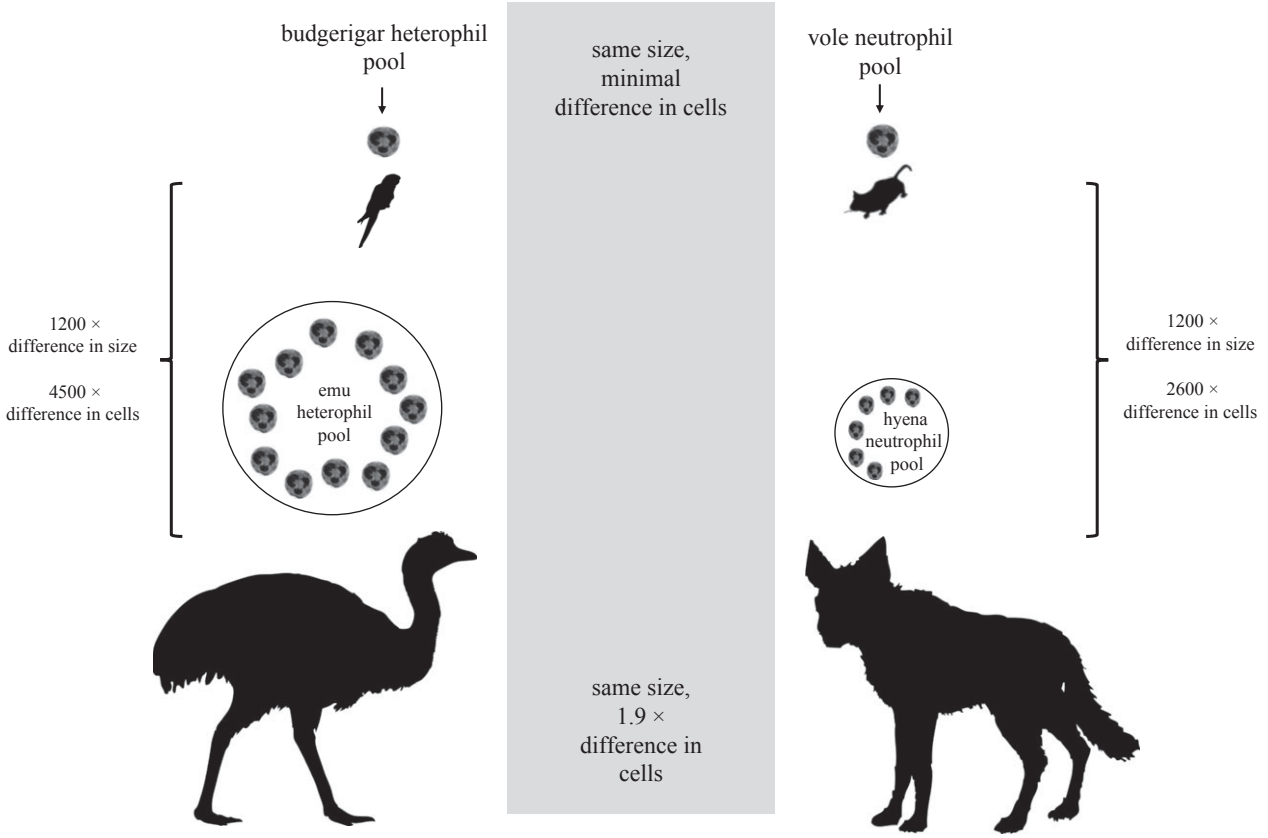

Figure 3. A visual summary of the heterophil/neutrophil concentration scaling differences between birds and mammals. Total heter-/neutrophil values were calculated using the intercepts and slopes from the mass-only models for birds and mammals, published body masses, blood volumes (approx. 9% total body mass [9,58,59]) and blood density values [35]. We multiplied the estimated total blood volume by the number of cells per litre of blood to extrapolate cell concentrations to whole organism total cell values. The shaded comparisons highlight that the heter-/neutrophil concentrations in a budgerigar and vole should be similar, but that an emu would circulate 1.9-fold more granulocytes than a striped hyena.

[19] and birds [27]). Previously, we argued that these patterns might have evolved because large organisms traverse greater distances and have higher surface area for infection while also experiencing disproportionate disadvantages relative to their parasites [4,6]. Especially for bacteria and viruses, replicative and evolutionary advantages of infective adversaries might have forced large hosts to evolve especially robust, generic constitutive defences [8,47,48]. Small hosts, by contrast, might circulate comparatively fewer of this cell type as a bet-hedge against such risks (given their inherently shorter lifespans), perhaps because they remain somewhat more capable of keeping pace in arms races with parasites because of their life-history strategy or because their life history generally favours reproduction over longevity. Future studies should strive to test these hypotheses, but at the same time, attempts should be made to resolve directly how body mass interacts with life-history traits to influence interspecific variation in immunity. As above, life-history traits have been claimed to drive immune variation among species and populations, but almost never have body mass effects on life history been disentangled from body mass [7]. Among the bird species studied here, we found some correlations between body mass and life-history traits (electronic supplementary material, figure S3). To understand why hypermetric scaling exists at all, it will be important to study body mass and life-history traits together, as large organisms tend to become large via long developmental periods and subsequent selection for low rates of mortality [12].

(c) Why do avian heterophils scale so steeply with body mass?

Birds and mammals differ in many ways, which might explain why heterophil concentrations in birds scale more steeply than neutrophil concentrations in mammals. For instance, birds are much better able to mitigate the costs of oxidative damage than mammals, which might be one way they achieve relatively longer lifespans than mammalian species of similar size [56,57]. The majority of such differences between classes, however, would be expected to influence only the *intercept* terms in the models we evaluated, not the slopes. In other words, whereas similar-sized birds tend to live longer than mammals [9], if such a difference influenced leucocyte concentrations, its effects would be expected to do so consistently across body masses, not disproportionately at large sizes as we observed. For most differences between birds and mammals (i.e. air sacs versus lungs for breathing, egg-laying versus live birth, organ mass, blood circulation/volume and many more), differences in avian heterophil concentrations would be expected to be consistently different from mammalian neutrophils across all body masses. Here, we observe no difference in the intercepts between bird heterophils (intercept, CI = 0.11, −0.09–0.31) and mammal neutrophils (intercept, CI = 0.15, −0.09–0.42 [24]). Our discovery of a fairly striking difference in the *steepness* of scaling between avian and mammalian granulocytes and mass was unexpected; *b* for birds in our mass-only model (model 3) was

approximately 1.5 × greater than the mammalian *b* estimated from a comparable model.

When data are plotted on log-log scales (figure 2*a*,*b*), their biological significance is often obscure, so to convey better the consequences of this difference, we produced conceptual figure 3 using species at opposite extremes of our body mass distribution. Using the equation from model 3, with published values for blood density and volume for birds [9,35,60], we calculated total whole-animal heter-/neutrophil values for species. We estimated that an emu (*Dromaius novaehollandiae;* 34 200 g) circulates 4.5 k-fold more heterophils than a budgerigar (29 g), although their body masses differ by just 1.18 k-fold. To capture the consequences of this allometric difference from what we observed previously in mammals [19], we tallied the number of cells in avian and mammalian species of the same size. We found that whereas a common vole (*Microtus arvalis*) and a budgerigar would maintain roughly the same number of granulocytes in their blood, an emu would circulate approximately 1.9-fold more heterophils (2.9×10^{19} cells) than a striped hyena (*Hyaena hyaena*) would circulate neutrophils (1.5×10^{19} cells; figure 3*c*).

Our approach cannot reveal *why* large birds circulate so many more heterophils than large mammals circulate neutrophils, but there are a few conspicuous possibilities. The first is that the two cell types are not functionally similar after all, and for some reason, avian heterophils might require disproportionate compensation for less effective function via increases in cell number as birds increase in size. The second possibility involves the mode of locomotion. Powered flight has evolved independently in birds and in the only groups of flying mammals, bats. It is plausible that the physiological constraints associated with the evolution of flight impacted aspects of immunity (e.g. hollow bones, lightened skeleton). One way to test this hypothesis would be to investigate neutrophil scaling in bats; such research has occurred, but allometric slopes were not estimated [50]. Of course, though, the largest birds do not fly, so flight alone is probably not the reason. A third possibility entails the lack of lymph nodes and leucocyte pools in birds. Because birds lack defined lymph nodes, they might not be able to shuttle heterophils as efficiently around their bodies, particularly at large body sizes, as mammals can do for neutrophils. Until we know how lymphoid tissue is organized relative to body mass in birds, we can only speculate that it might scale more hypometrically than mammals. In mammals, we know that myelocytes (leucocyte progenitors) can reside in a 'lazy pool', and mature neutrophils can persist in a 'rapid mobilizable pool' [28]. The existence of heterophil pools in birds is unknown, but as we proposed for lymphoid tissue, steep hypometric scaling of the size or numbers of these pools could explain hypermetric scaling of heterophils in birds. A final possibility for scaling disparities between classes is how physical barriers scale with size. Bird feathers and mammalian fur both scale hypometrically [9], which might make large mammals and birds more susceptible to infection [6] and generally necessitate granulocyte hyperallometries. Perhaps the more negative *b* for avian skin exists because of its thinness and simplicity, which is distinct from mammalian skin [61].

We are far from resolving why birds require so much more heterophil-mediated protection at large body sizes, but we encourage additional work on the topic. It is also obscure whether the patterns we detected truly represent evidence for the safety factor hypothesis, so we advocate that future work try to address this open question. Such insight will be useful for resolving how, and why, body size affects disease ecology and evolution. We expect that additional mechanistic studies will be useful, particularly the development of more precise tools for distinguishing lymphocyte classes or methods that better capture functional immune variation (e.g. direct control of infections) among species.

Ethics. All leucocyte data used in this study were obtained from the ZIMS database maintained by Species360, which collects information from healthy, captive animals held at Association of Zoos and Aquariums (AZA) accredited facilities. Life-history data were obtained from published databases.

Data accessibility. Data used in this analysis are available in the electronic supplementary material and from the Dryad Digital Repository: https://doi.org/10.5061/dryad.s7h44j13t [62].

Authors' contributions. E.C.R., L.B.M. and C.J.D. conceived the idea; C.J.D. oversaw the collection of the data; E.C.R. and C.J.D. conducted the statistical analysis; E.C.R., L.B.M. and C.J.D. wrote and revised the manuscript.

Competing interests. The authors have no competing interests to declare.

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