Revisiting the question of nucleated versus enucleated erythrocytes in birds and mammals.

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Abstract

Erythrocyte enucleation is thought to have evolved in mammals to support their energetic cost of high metabolic activities. However, birds face similar selection pressure yet possess nucleated erythrocytes. Current hypotheses on the mammalian erythrocyte enucleation claim that the absence of cell organelles allows erythrocytes to 1) pack more hemoglobin into the cells to increase oxygen carrying capacity and 2) decrease erythrocyte size for increased surface area–to-volume ratio, and improved ability to traverse small capillaries. In this article, we first empirically tested current hypotheses using both conventional and phylogenetically informed analysis comparing literature values of mean cell hemoglobin concentration (MCHC) and mean cell volume (MCV) between 181 avian and 194 mammalian species. We found no difference in MCHC levels between birds and mammals using both conventional and phylogenetically corrected analysis. MCV was higher in birds than mammals according to conventional analysis, but the difference was lost when we controlled for phylogeny. These results suggested that avian and mammalian erythrocytes may employ different strategies to solve a common problem. To further investigate existing hypotheses or develop new hypothesis, we need to understand the functions of various organelles in avian erythrocytes. Consequently, we covered potential physiological functions of various cell organelles in avian erythrocytes based on current knowledge, while making explicit comparisons to their mammalian counterparts. Finally, we proposed by taking an integrative and comparative approach, using tools from molecular biology to evolutionary biology, would allow us to better understand the fundamental physiological functions of various components of avian and mammalian erythrocytes.

Introduction

Endothermic animals are capable of generating heat metabolically to maintain relatively high and stable body temperature (34) and engaging in energetically demanding behaviours that require high aerobic capacity (3). To sustain metabolically expensive activities, an effective oxygen transport system is essential. One way high oxygen transport capacity can be achieved is by modifying blood parameters such as increasing hemoglobin (Hb) levels, enhancing blood flow, and increasing diffusion efficiency of oxygen from erythrocytes to tissues (73, 83). Consequently, it has been proposed that the enucleated erythrocytes in mammals (83), might be responsible for an efficient oxygen transport system. Traditionally, enucleation only refers to the extrusion of nucleus, however, we expand the idea of enucleation to include all cellular organelles since the extrusion of all organelles are tightly coupled and accompanied (42). This phenomenon is unique among vertebrates, as all mammals, including monotremes, placental, and marsupial mammals all possess enucleated erythrocytes. With the exception of a few salamander species (67), other major vertebrate taxa including birds, reptiles, fishes, and amphibians all possess predominantly nucleated erythrocytes in circulation. Furthermore, with the exception of mammalian erythrocytes, all mammalian somatic cells are nucleated. This prompts the question of how mammalian erythrocytes function as cells without major cell organelles.

Some popular hypotheses for the evolution of enucleated erythrocytes in mammals state that 'extrusion' of cell organelles allows these cells to either pack more Hb into the erythrocytes to increase oxygen carrying capacity, or decrease erythrocyte size for increased surface area–to-volume ratio, facilitating increased gas exchange efficiency, and improving the cell's ability to traverse small capillaries (42, 83). Extrusion of cell organelles from mammalian erythrocytes is controlled via tightly regulated molecular and cellular pathways, starting with chromatin condensation and increase in Hb concentration during erythroblast differentiation, nucleus polarization and nuclear extrusion leading up to reticulocyte formation, and eventually extrusion of mitochondria and other cell organelles during the final maturation stage to form enucleated erythrocytes (42, 65). Together, these observations prompt important questions regarding evolutionary and physiological implications of erythrocyte enucleation.

Although taxonomic and allometric differences in blood parameters within mammals have been analyzed phylogenetically (73), the idea of enucleated erythrocytes having more Hb per cell volume has not been tested using a phylogenetic framework (29, 83). Although major vertebrate taxa besides mammals all possess predominantly nucleated erythrocytes, birds (as opposed to fishes, amphibians, and reptiles) provide a key taxon for comparison to mammals. As the only two endothermic classes in the world, birds evolved endothermy independently of mammals (31, 58). Birds also have higher body temperature and face presumably similar selection pressure compared to mammals. Despite these similarities to mammals, birds possess erythrocytes with not just a nucleus but also other cell organelles (27, 88). We propose that by understanding the function of organelles in avian erythrocytes and comparing erythrocytes between birds and mammals, we can provide some insights into how mammalian erythrocytes

function without cell organelles, as well as why birds retained cell organelles in their erythrocytes.

In this essay, we will first discuss the current hypotheses that attempt to explain the evolution of enucleated erythrocytes in mammals. Then, we will test these hypotheses empirically using published hematological values from the literature (78) and provide evidence that demonstrate why those hypotheses do not apply to evolution of avian erythrocytes. We restrict our analyses to birds and mammals partly because we have the most comprehensive hematological and morphological dataset from those two classes. More importantly, it has been shown previously that ectotherms such as amphibians, fishes, and reptiles have larger erythrocytes and lower mean cell Hb content than birds and mammals (29, 83). We believe that making specific hypotheses is nearly impossible without basic understanding of the differences between avian and mammalian erythrocytes, why those differences exist, and the physiological functions of cell organelles in erythrocytes. Therefore, we will discuss potential physiological functions of various cell organelles in avian erythrocytes based on current knowledge, and their implications at the organismal level. Additionally, we will make explicit comparisons to their mammalian counterparts. Finally, we will end by proposing potential paths for future studies that would allow us to better understand fundamental physiological functions of various components of avian and mammalian erythrocytes.

Existing hypotheses

Hypothesis 1: Extrusion of nucleus and cell organelles allows mammals to pack more Hb into erythrocytes.

Arguably the most accepted hypothesis for why mammalian erythrocytes extrude cell organelles is that extrusion occurs to allow a higher density of intracellular Hb. This popular hypothesis is included in many biology related books, from educational to general biology (75), physiology (80), to anatomy textbooks (60), often without presentations or citations of empirical data. Interestingly, previous studies that investigated Hb concentrations in avian and mammalian erythrocytes using traditional analysis indicated that mean cell Hb concentration (MCHC) was not different between birds and mammals when cell volume is accounted for (33, 78, 83). To our knowledge, the difference in MCHC between avian and mammalian erythrocytes has not been investigated in a phylogenetic framework.

Therefore, we obtained blood parameters data of birds (n=181 species from 23 orders), mammals (n=194 species from 20 orders) from two previous studies (S5, https://doi.org/10.6084/m9.figshare.13028228) (23, 78), as well as a phylogenetic tree of the species included in the analysis from the timetree for the tree of life (Supplementary Figure S2) (35). At the same time, we also included 32 species of bony finshes together with birds and mammals to verify our analysis (Supplementary Table S6). Using this dataset, we tested whether MCHC differs between birds and mammals using both ordinary least squares (OLS), and phylogenetic generalized least squares (PGLS) regressions, with class (i.e. , aves or mammalia) as a predictor of MCHC and body mass and mean cell volume (MCV) as covariates (see Supplementary S1 for detailed methods, https://doi.org/10.6084/m9.figshare.13028228; R

script used in this study is available at https://doi.org/10.6084/m9.figshare.13028228). Neither OLS nor PGLS indicated that MCHC differ significantly in birds and mammals (OLS: $F_{1, 370} = 0.865$, p = 0.353; PGLS: $F_{1, 370} = 0.003$, p = 0.957; Figure 1 A,B; Supplemtary table S4). Consistent with previous studies (33, 78, 83), our findings demonstrate that even though mammalian erythrocytes do not possess nucleus and other cell organelles, no difference in Hb concentration were observed between these cells in birds and mammals. Moreover, the addition of bony fishes as an outgroup did not alter the findings of our analysis for birds and mammals. According to OLS, MCHC was similar between birds and mammals but lower in bony fishes ($F_{1, 401} = 84.74$, $p < 0.01$; Supplementary Figure S3A). However, PGLS indicated that MCHC did not differ significantly in fishes, birds, and mammals ($F_{1, 401} = 0.04$, p = 0.96; Supplementary Figure S3B).

Consequently, although the hypothesis of enucleated erythrocytes allowing for higher concentration of intracellular Hb could possibly explain the evolution of enucleated mammalian erythrocytes, the hypothesis does not apply to the evolution of avian erythrocytes. Part of why birds have similar MCHC to mammals can be attributed to the presence of Hb within the nucleus of avian erythrocyte (36). Rather than extruding the nucleus from their erythrocytes during the erythroid maturation process like mammals do, birds seem to have evolved the means to maintain high MCHC through the integration of Hb into the nucleus of their erythrocytes (77, 104). Interestingly, the expression of multiple hemoglobin isoforms with different isoelectric point may also be a mechanism to increase MCHC (98). Some birds express both Hb isoform A and D, but most mammals express only Hb isoform A (71). In short, these observations likely indicate different solutions employed by birds and mammals for a common problem.

Phylogenetic analyses on MCHC levels between ectotherms and endotherms have not been conducted. However, previous studies have shown that birds and mammals generally have higher MCHC than amphibians, reptiles, and fishes (33, 83), suggesting that regardless of evolutionary strategies, high MCHC might be needed to satisfy the increased oxygen demands of endothermic animals. Consequently, the adaptation of high MCHC is typically associated with the evolution of endothermy. Future studies comparing MCHC levels between ectotherms and endotherms using a phylogenetic framework are desperately needed to draw better conclusions.

Hypothesis 2: Extrusion of cell organelles in mammalian erythrocytes minimizes cell volume, thereby increasing surface area–to-volume ratio, facilitating increased gas exchange efficiency, and allowing erythrocytes to traverse small capillaries.

Only a handful of studies have directly compared erythrocyte size between birds and mammals, and all of these studies employed conventional statistical methods without taking phylogeny into account. For instance, Scanes (78) found that MCV was higher in birds than mammals. Here, we employed both OLS and PGLS to compare MCV between birds and mammals with body mass as covariates. Similar to previous findings (78), OLS showed that MCV was higher in birds than in mammals ($F_{1, 371} = 561.799$, $p < 0.01$), however, the significant

difference was lost when we corrected for phylogeny ($F_{1, 371}$ = 0.390, p = 0.532; Figure 1 C,D; Supplemtary table S4). This indicates that birds indeed have bigger erythrocytes than mammals, but the difference is largely due to phylogeny. Similar to our findings concerning MCHC, the addition of the bony fishes as an outgroup did not alter the findings of our MCV analysis for birds and mammals. OLS showed that MCV was higher in birds and bony fishes than in mammals ($F_{1, 402}$ = 317.69, p < 0.01; Supplementary Figure S3C), however, the significant difference was lost when we corrected for phylogeny ($F_{1, 402}$ = 0.26, p = 0.76; Supplementary Figure S3D). Although there are no empirical data explaining the physiological and evolutionary implications of the taxonomic difference in avian and mammalian erythrocytes, a few hypotheses have been proposed.

First, it has been proposed that smaller cell volume allows erythrocytes to transverse small blood capillaries. Snyder & Sheafor (83) compared erythrocyte size and capillary diameter in nine species of amphibians and a mammal (rat) and found positive correlation between erythrocyte width and capillary diameter, leading to the conclusion that erythrocyte size is limited by capillary diameter. However, we need to be cautious about their findings due to the limited number of species that were included in their comparison. Additionally, a study looking at the evolution of enucleated erythrocytes in several clades of the salamander family Plethodontidae suggested that enucleated erythrocytes evolved in conjunction with their large genomes and miniaturized or attenuated body forms (67). Similarly, it has been shown that hummingbirds with larger genome also tend to have larger erythrocytes (30). However, to our knowledge, similar comparisons have not been made between birds and mammals. Additionally, we argue that aside from capillary diameter, elasticity of capillary wall and deformability of erythrocytes also needs to be considered when determining the ability of erythrocytes to transverse blood capillaries (64, 96). Elasticity of systemic capillaries have not been measured in birds. However, avian pulmonary capillaries exhibit near-rigid properties compared to mammalian capillaries even when subjected to extreme changes in transmural pressure (96, 99). Larger erythrocytes are also more deformable than smaller ones (83). Taken together, this observation suggests that neither capillary diameter nor capillary elasticity are the key determinants of erythrocyte size.

Second, smaller erythrocyte size has also been proposed to increase surface area–to-volume ratio, which in turn would promote relatively fast O_2 uptake and unloading kinetics to increase gas exchange efficiency (36). While small erythrocyte size might increase O_2 uptake velocity (36), avian erythrocytes exhibit a suite of morphological and physiological adaptations that enable high gas exchange efficiency. For instance, birds have higher ratio of capillary surface area to muscle fibre surface area in their flight muscle, compared to locomotory muscles of mammals (61, 62). Similarly, their heart and brain also have higher densities of capillaries compared to mammals (22, 79). Compared to mammals, birds also have much thinner and more uniformed blood-gas barrier (99), which could in turn lead to faster O_2 uptake and unloading kinetics. Consequently, we argue that diffusion of O_2 kinetics in various tissues might be higher in birds than in mammals despite their erythrocytes being larger. Besides the size of erythrocytes, the shapes of erythrocytes, which are different for birds and for mammals,

could also influence gas exchange efficiency (27). Therefore, while it is possible that mammals evolved small erythrocytes to maximize O_2 uptake and unloading kinetics, birds evolved different mechanisms to achieve the same evolutionary outcomes.

Third, larger avian erythrocytes has also been tied to genome size evolution, which posits that animals with larger genome would have larger cells, including erythrocytes (29, 83). Even though positive relationship between genome size and erythrocyte size has been detected within each of the vertebrate classes, however, the relationships between classes were still unknown (29). Kapusta et al. (49) took advantage of available genome sequences for a wide range of species and compared genome sizes of birds and mammals. They showed that genome sizes of birds (0.96 - 2.2 Gb) and mammals (1.6 - 6.3 Gb) overlap. Nevertheless, as we discussed in previous paragraphs, birds do have larger erythrocytes than mammal. This suggests that extrusion of cell organelles, which has been hypothesized to accompany a decrease in genome size, might not be the reason for evolution of smaller erythrocytes in mammals (49). Rather, smaller erythrocytes in mammals could merely be a consequence of extrusion of cell organelles.

Taken together, while it is very possible that extrusion of cell organelles from erythrocytes decreased cell volume, which maximized O_2 uptake and unloading kinetics in mammals, this hypothesis fails to explain why and how birds retained cell organelles in their erythrocytes yet maintained efficient O_2 uptake and unloading kinetics. Although previous studies have shown that birds and mammals generally have smaller erythrocytes compared with other ectothermic classes (29, 33, 83), phylogenetic corrected analyses between endotherms and ectotherms are needed to examine if smaller MCV is indeed associated with the evolution of endothermy.

Hypothesis 3: Extrusion of mitochondria and endoplasmic reticulum (ER) decreases oxidative stress

Mitochondria, ER and other cellular organelles could produce large amount of reactive oxygen species (ROS) in cells (11, 93). Recently, it has been proposed that the extrusion of cell organelles from erythrocytes could allow organisms to avoid potentially harmful effects of ROS generated by these organelles (106). Particularly, the idea of mitochondria extrusion in mammalian erythrocytes has gained much attention for the alleged consequence of reduced production of damaging ROS (106). This is an attractive hypothesis given that human erythrocytes generate extensive free radicals even without mitochondria, necessitating co-evolution of an arsenal of protective antioxidant molecules and enzymes (55). However, existing data suggest that birds exhibit lower levels of blood oxidative damage in comparison with mammals, despite retaining both functional mitochondria and nuclei in their erythrocytes (14, 44). It is still unknown whether erythrocytes in mammals would experience higher oxidative damage if mitochondria and ER were retained, but this suggests that the presence of mitochondria and ER in erythrocytes is not the only factor that regulates ROS production and oxidative stress.

Other than ROS production, mitochondria can also release cytochrome C which ultimately induces apoptosis in dysfunctional cells (51). Extrusion of mitochondria in

mammalian erythrocytes could serve as a protective mechanism against erythrocyte apoptosis. If this is true, we would expect mammalian erythrocytes to have longer lifespan (i.e. lower turnover rate) than their avian counterpart. Indeed, avian erythrocytes have shown to exhibit higher (about 40%) turnover rate than mammalian erythrocytes (27, 76). This suggests that there could be a functional link between the presence of mitochondria in erythrocytes and shorter erythrocyte lifespan in birds than in mammals. However, this idea has never been tested empirically. Furthermore, there is some evidence suggesting that despite being enucleated, mammalian erythrocytes are capable of self-execution by both cell-specific mechanism and that resemble apoptosis of nucleated cells, often referred to as eryptosis or quasi-apoptosis (32).

Synthesis of existing hypotheses

In short, although the three main hypotheses for enucleation of erythrocytes in mammals are widely accepted, and repeated, in the literature, they fail to explain why and how birds retained nucleus and cell organelles in their erythrocytes yet managed to evolve metabolically active machinery similar to mammals. To further understand why enucleation of erythrocytes evolved in mammals but not in birds, and to generate new testable hypotheses, it is important to first understand the fundamental functions of the various cell organelles in avian erythrocytes, and understand the physiological and evolutionary consequences of these organelles. As we mentioned before, one way to investigate how mammalian erythrocytes function without cell organelles and why birds evolved to retain cell organelles in their erythrocytes is by comparing erythrocytes of birds and mammals. Specifically, some fundamental questions that need to be addressed are: Are organelles in avian erythrocyte functional? If organelles in avian erythrocytes are functional, what are their functions? Do avian erythrocyte organelles have similar functions as organelles of cells in other tissues? Do what we see in avian erythrocytes reflect physiological state of other tissues or the whole body? If cell organelles in avian erythrocytes are indeed crucial components of normal physiological function, how do mammals operate without any cell organelles in their erythrocytes? There is a significant knowledge gap regarding all of the important questions above and they require further study. In the next section, we will discuss potential functions of the organelles of avian erythrocytes based on current knowledge.

Potential roles of cell organelles in avian erythrocytes

Nucleus and ribosome

Erythrocytes play a number of physiological roles aside from merely participating in gas exchange, and this is true even for enucleated mammalian erythrocytes (55, 85). The presence of nucleus and ribosome in erythrocytes could allow erythrocytes to synthesize proteins and molecules *de novo* in response to stressors and external stimuli.

It has been assumed that avian erythrocytes follow a highly conserved role as observed in mammalian erythrocytes with total inactivity in transcription and translation (104). From a ground-breaking study conducted nearly half of a century ago that found “messenger-like” RNA

in avian erythrocytes' nucleus (105), to recent studies that investigated transcriptome of avian erythrocytes using modern RNA-seq techniques (66, 77), it appears that there is at least some level of DNA transcription activity in the nucleus of avian erythrocytes. Although proteomic studies have not been conducted on erythrocytes of avian species, nucleated erythrocytes from other classes have shown protein level changes under different environmental and/or physiological conditions (68).

Recently, a set of biological processes related to immune function such as phagocytosis (72), antigen presentation (16), and interleukin-like production (16, 66) has been reported in nucleated erythrocytes from different organisms. These observations, together with studies on transcriptome of avian erythrocytes and antiviral functions, indicate that nucleated erythrocytes could have important role in immune function (which involves significant amount of protein synthesis) in birds (69, 86). However, it is still unclear whether nucleus and ribosomes are fully functional in avian erythrocytes. It is also worth noting that transcriptomic and proteomic studies have been conducted on mammalian erythrocytes (16, 28), and they generally found that enucleated erythrocytes can employ different strategies against toxins and pathogens and have protective effects on various diseases (37, 43, 94).

Unlike mammals, which are capable of storing erythrocytes in spleen and can rapidly up-regulate hematocrit and Hb in response to stimulus such as exercise (102), avian spleen are not capable of storing erythrocytes (46). Therefore, one possible way for birds to modulate the levels of Hb, a major oxygen transport protein, is by repairing or synthesizing Hb *de novo*. It is plausible that the nucleus and other organelles would work in conjunction to synthesize and repair Hb *de novo* in avian erythrocytes. It should be noted however, that there is currently no evidence that avian erythrocytes are capable of repairing or synthesizing Hb *de novo.*

Supporting evidence indicates that unlike mammals, erythrocyte production and Hb synthesis are regulated independently in birds, whereas these are coupled in mammals (27). Moreover, transcriptomics study indicated that hematopoiesis pathway is regulated in avian erythrocytes (48). Researchers proposed that Hb synthesis in avian erythrocytes occurs on ribosomes located either at the outer nuclear membrane (18) or cytoplasm (48). Furthermore, nucleated erythrocytes in fish are capable of synthesizing Hb *de novo* (50, 84). This evidence suggests that *de novo* synthesis and repair of Hb could happen in avian erythrocytes.

The ability to synthesize and repair Hb and potentially other proteins could be one of the 'evolutionary benefits' of retaining cell organelles in mature avian erythrocytes. However, if synthesis and repair of Hb is a vital process in avian erythrocytes, we should expect the lifespan of avian erythrocytes to be longer than their mammalian counterparts. However, as discussed in the section above, birds have actually been shown to have much higher erythrocyte turnover rate than mammals (27), but this could simply be due to the fact that avian erythrocytes are metabolically active whereas mammalian erythrocytes are metabolically inactive (106). RNA isolated from mature avian erythrocytes also do not match cDNA globin sequences in reticulocytes (100), suggesting that these RNAs are product of mature erythrocytes rather than leftover remnants of immature reticulocytes. Hence, it is still inconclusive as to whether avian erythrocytes could synthesize Hb *de novo*.

In short, data from past studies showed promising evidence that the nucleus (and ribosome) in avian erythrocytes could transcribe and translate genes in response to external stimuli. Mammals on the other hand, could simply rely on the plasma membrane proteins of somatic cells (e.g. P-gp) (94), and their ability to store and release erythrocytes from the spleen to deal with environmental stressors such as immune challenge and hypoxia. Studies on stressors other than immune challenge should be conducted to investigate whether nucleus and ribosomes of avian erythrocytes are fully functional, compared to nucleus and ribosomes of other cell types.

ER and Golgi apparatus

The ER and Golgi apparatus play central roles in the management of the folding and transport of synthesized protein. Similar to hematopoietic stem cells in mammals (81), nucleated erythrocytes in fish have been shown to exhibit ER stress and unfolded protein responses (52, 56). If indeed nucleated erythrocytes are capable of synthesizing and repairing proteins (see previous section on nucleus and ribosome), the ability to undergo unfolded protein response to deal with high protein load and misfolded proteins in the ER is crucial to preserve cellular function (8, 9).

The ER also plays important roles in the management of intracellular calcium which acts as a second messenger in cells. The ER employs sarco-endoplasmic reticulum Ca^{2+} ATPase pumps to control the transient release and re-uptake of Ca^{2+}. Previous studies have demonstrated that the sarcoendoplasmic reticulum Ca^{2+} ATPase in nucleated erythrocytes of lizards serves to regulate intracellular calcium pools (1, 4). Elevated intracellular levels of Ca^{2+} as a result of Ca^{2+} uptake by sarcoendoplasmic reticulum Ca^{2+} ATPase could also trigger erythrocyte vesiculation, a process in which erythrocyte loses membrane surface and Hb content, leading to decreased surface-to-volume ratio (6). Erythrocyte vesiculation has been suggested as a physiological process to protect the cell from its removal from the circulation (38). Furthermore, some of the toxin metabolism and detoxification functions of erythrocytes could be attributed to the ER and its associated enzymes (54, 94).

In sum, the ER's ability to serve as quality control system of avian erythrocytes, regulate intracellular Ca^{2+} levels, and possibly aid in toxin metabolism could be beneficial for erythrocyte function. Although it has been shown that other organelles such as Golgi apparatus, phagosomes, exosomes play crucial roles in the maturation of avian erythrocytes (47), their functions in mature avian erythrocytes warrant further investigation.

Mitochondria

Mitochondria in avian erythrocytes are perhaps the organelle that has attracted the most research attention. From pioneering bioenergetics studies where avian erythrocytes were used to demonstrate adenosine triphosphate (ATP) production during respiration (20, 21), to recent studies where avian erythrocytes were shown to have mitochondria that respond to various mitochondrial complex inhibitors (88, 90), there is strong evidence that avian

erythrocytes do indeed have functional mitochondria. Interestingly, a recent paper has proposed that avian erythrocyte mitochondria might contribute to themogensis (70). However, the exact physiological roles and implications of mitochondria in erythrocytes (i.e. whether their functions are similar to other somatic cells) are currently unknown.

As the energy hub of cells, one of the major roles of mitochondria is ATP production. In birds, erythrocytes have been shown to contain more intracellular ATP than their mammalian counterparts (63), implying a role for energy production by mitochondria in avian erythrocytes. However, for mammals, only a small amount of ATP is required to maintain erythrocyte forms and functions under normal condition, which mainly involve maintaining osmotic gradient (38). Furthermore, even in mammalian erythrocytes where all ATP production comes exclusively from glycolysis, erythrocytes with malfunctioned glycolytic enzymes usually do not show altered morphology (39, 53). This indicates that perhaps the presence of mitochondria in avian erythrocytes is not purely for energetics purposes. If that is true, what potential roles do ATPs produced by mitochondria in avian erythrocytes play at the cellular and whole-organism levels?

In cells, protein and RNA synthesis have the highest demand for ATP, much more than can be produced via glycolysis (10). Consequently, ATP produced by mitochondria in avian erythrocytes could help fuel DNA transcription and translation, as discussed above (66, 68, 77). Following the hierarchy of ATP-consuming processes in cells, intracellular ion cycling is the next major ATP consumer. Mitochondria in erythrocytes could serve to better regulate ATP levels which on one hand could help maintain osmotic pressure of erythrocytes, and on the other hand, initiate erythrocyte deformation when needed. Although a previous study showed that avian erythrocytes are hardly deformable compared to mammalian erythrocytes (25), the study was conducted using an *in vitro* setup and erythrocytes were tested in environments that were different from the animals' physiology. Erythrocyte deformability can be controlled via ion handling by pumps and passive transport pathways (12, 38), proteolytic activity of Ca^{2+}-dependent protease calpain (6), and mutations and structural integrity of each element of the membrane architecture (26), all of which are tightly regulated by ATP levels and redox state of the cells.

ATP produced by mitochondria within avian erythrocytes could also help regulate Hb–O_2 affinity. Organic phosphates, such as 2,3-diphosphoglycerate (DPG) and ATP, could serve as allosteric effectors in erythrocytes (2, 59). Phosphate binding reduces Hb–O_2 affinity by shifting the conformational equilibrium in favor of the low-affinity T-state; this allosteric transition in quaternary structure promotes O_2 unloading to the cells of respiring tissues (2). In mammals, Hb–O_2 affinity is modulated predominantly by DPG, and to some extent ATP and metabolites produced via anaerobic glycolysis (24, 92). The potential downside of relying on ATP produced via glycolysis is that glycolysis typically promotes acidity in erythrocytes, and a drop in cellular pH could in turn decrease Hb–O_2 affinity (7). Although inositol pentaphosphate is the main regulator of Hb–O_2 affinity in birds (97), avian erythrocytes could potentially also respond to environmental hypoxia by modulating erythrocyte concentrations of other nucleotide triphosphates such as ATP and GTP produced by the mitochondria to adjust and fine tune Hb–O_2 affinity to enhance O_2 uptake and/or unloading (41, 97). Regulating ATP levels using

mitochondrial OXPHOS has the advantage of maintaining intracellular pH level while modulating Hb–O_2 affinity (7). Despite the supposed benefit of modulating Hb- O_2 affinity using ATP produced by mitochondrial OXPHOS, it remains to be seen whether birds actually acclimatize to environmental hypoxia via changes in red cell organic phosphate concentrations.

Other than interacting with Hb to regulate oxygen affinity during hypoxia, ATP produced from mitochondria in avian erythrocytes could also be released by erythrocytes to signal vasodilation of the endothelium (15, 19). Owing to the absence of intracellular organelles, mammalian erythrocytes were considered the ultimate example of a cell system where ATP release occurs mostly if not exclusively via hemolysis (82). With the presence of mitochondria and other cellular organelles in avian erythrocytes, several channels such as voltage-dependent anion channels could be used for ATP release, rather than relying on hemolysis. This would allow for rapid ATP release to induce vasodilation without sacrificing total level of erythrocytes under hypoxic conditions. Furthermore, this could presumably protect the organism from negative effects of having free Hb in circulation and oxidative stress resulting from hemolysis (108).

Erythrocytes are also under tight redox control (55). Unlike mammalian erythrocytes, avian erythrocytes possess mitochondria that could serve as providers of electron donors, reservoir of antioxidants, and a system for maintaining ion homeostasis (57, 88). There are multiple sources of oxidation in erythrocytes, including high levels of molecular O_2 bound to Hb, as well as high levels of iron which is a potent catalyst of ROS production via the Fenton reaction (55). In avian erythrocytes in particular, the presence of mitochondria also results in higher ROS production, compared to mammalian erythrocytes (106). Formation of methemoglobin (metHb) by oxidation of the ferrous heme (Fe^{2+}) iron contained in the prosthetic group of Hb to ferric (Fe^{3+}) heme causes a dramatic decrease in Hb–O_2 affinity. Thus, to preserve its functionality, Hb needs to be maintained in the reduced state (55). In fact, it has been proposed that mammalian erythrocyte extrudes cell organelles to minimize oxidative stress (106). However, a study comparing ROS production and oxidative damage in erythrocytes of zebra finch (*Taeniopygia guttata*) and house mouse (*Mus musculus*) showed that although erythrocytes of zebra finch exhibited higher ROS production, oxidative damage was actually lower compared to erythrocytes of house mouse due to high levels of antioxidants, suggesting that high ROS production does not always lead to high oxidative damage (88). It is also important to note that mitochondria may not be the major site of ROS production in different cell type (101), and considering the abundance of Hb in erythrocytes, autoxidation of Hb is likely the most abundant source of ROS in erythrocytes (55).

Malfunction of antioxidant defense and/or conditions of increased oxidant production have severe consequences for cells (including erythrocytes) at a subcellular level. Every 24 h, 3% of Hb undergoes autoxidation, producing metHb and superoxide radical anion (55). However, only 1% of Hb is present in the metHb (Fe^{3+}) state. Hb-Fe^{3+} can be reduced back to Hb-Fe^{2+} in a reaction catalyzed by the cytochrome b5 reductase using NADH as an electron donor (55). In avian erythrocytes, mitochondria are able to produce large amount of electron donor whereas mammalian erythrocytes can only depend on the pentose phosphate cycle as

the main source of reducing equivalents (55). The coordinated action of all antioxidant systems in avian erythrocytes presumably allows erythrocytes to be resistant against oxidation and serves as an efficient systemic redox buffering system. Therefore, it is possible that mitochondria in avian erythrocytes serve to better regulate redox status, reducing equivalents levels, and antioxidant capacities inside the cell which could protect cells from malfunctioning.

Given that flight is an extremely energetically demanding exercise, and that birds often have to fly at different altitudes with varying levels of oxygen (5, 79), it seems plausible that birds retain mitochondria in their erythrocytes to provide better control of the Hb-O_2 binding affinity. Furthermore, mitochondria and other cellular organelles could allow erythrocytes to be fueled by other substrates other than glucose (17). Additionally, it should be noted that although mitochondria in erythrocytes could serve many physiological roles, recent studies have shown relatively low abundance of mitochondria within avian erythrocytes (88, 90). Furthermore, there is also a major confounding factor in previous studies that measured erythrocyte mitochondrial function as they did not dissociate mitochondria from Hb (88, 90). Therefore, comprehensive studies on erythrocyte mitochondrial function and its regulation under different conditions must be conducted before jumping into any conclusion or inferring any physiological implications of mitochondria in avian erythrocytes.

Moving forward

The evolutionary implications of cell organelle extrusions in mammalian erythrocytes are much more complicated than previously thought. The existing and widely accepted hypotheses are largely based on observational studies that lack empirical support or understanding about basic physiological functions of organelles. Moving forward, to establish valid and testable hypotheses, we propose taking an integrative and comparative approach to studying the physiological and evolutionary implications of retaining versus extruding cell organelles from erythrocytes of birds and mammals respectively.

Taking an integrative and comparative approach will contribute to our understanding of the evolution of enucleation. Perhaps more importantly, it can provide important insights into many erythrocyte-related diseases in biomedicine. Mammals (including humans) actually possess nucleated erythrocytes but they can only be found either within five days of conception of human infants or in pathologic states (74). In humans, pathogenesis of dyserythropoietic diseases that cause abnormal erythrocyte formation such as myelodysplastic syndrome, leukemia and anaemia are often linked to nucleated erythrocytes (65). Given that birds function normally with nucleated erythrocytes, understanding the physiological implications of various cell organelles in nucleated erythrocytes could help provide novel and alternative approaches to the treatment of these diseases.

Compared to birds, the molecular and cellular mechanisms underlying maturation of erythrocytes in mammals have been studied much more extensively. The molecular and cellular processes of enucleation have been nicely reviewed in other articles (42, 65). This significant amount of knowledge was obtained mostly using genetically modified animals and *in vitro* models that involve culturing and differentiating erythroblasts *ex vivo* (42, 107). Primary cultures of chicken erythrocytes have recently been developed (45).The *in vitro* model would

allow us to conduct tightly controlled laboratory studies, as well as to investigate cellular processes governed by various organelles during erythrocyte maturation. For example, one could experimentally induce enucleation of avian erythrocytes by targeting the molecular pathways responsible for enucleation of mammalian erythrocytes (42), and studying the functional consequences of enucleation. Similarly, to investigate whether avian erythrocytes can synthesize and repair Hb *de novo*, one could either expose avian erythrocyte culture to hypoxia and/or treat the cells with erythropoietin and measure MCHC (103). These mechanistic studies would lay the foundation for further investigations at the organismal level.

At the organismal level, we can also gain significant insights into basic physiological functions of avian erythrocytes by conducting omics studies on avian erythrocytes exposed to various environmental and/or physiological conditions such as hypoxia and heat, as well as pathogens such as avian malaria and influenza (40), similar to what has been done in other systems (68). These omics studies could serve as pioneering steps to narrow down potential pathways in erythrocyte physiology. Capitalizing on insights gained from these omics studies, pharmaceutical and genetical approaches could then be employed to experimentally manipulate target genes and pathways to investigate their physiological implications at the organismal level.

Ideally, both *in vitro* and *in vivo* approaches should be employed simultaneously to study physiological function of various cell organelles in avian erythrocytes. Some eco-physiologists often use mitochondrial measurements from avian erythrocytes to infer animals' bioenergetics status at the organismal level (87–89). Although we understand the value of repeated measures and non-lethal sampling, simply assuming mitochondria in erythrocytes function similarly across other tissues could be misleading, especially given our limited understanding of mitochondria in avian erythrocytes. That being said, findings of positive correlations between mitochondrial measurements from erythrocytes and permeabilized pectoral muscle fiber, as well as repeatability of erythrocyte mitochondrial physiology across time are encouraging (87, 91). More experimental studies need to be conducted to firmly establish erythrocyte mitochondria measurements as biomarkers. Traditional mitochondrial function assessments involve measurements of respiration. This could be challenging to execute in erythrocytes since Hb serves as a large reservoir to bind and release cellular oxygen. Therefore, other approaches such as mitochondrial membrane potential sensitive fluorescent probe could be a better alternative to evaluate mitochondrial function in avian erythrocytes.

Admittedly, a large portion of this review surrounds discussion of physiological roles of mitochondria in erythrocytes. This is in part due to the nature of the field with the majority of the studies focusing on mitochondrial functions of avian erythrocytes. Studies on physiological functions of other organelles in avian erythrocytes are scarce. An obvious first step is to determine whether those organelles are truly functional in avian erythrocytes. This can be done by subjecting erythrocytes to different stressors or inhibitors and studying the corresponding physiological responses. For example, ER stress inducers or inhibitors could be used to treat avian and mammalian erythrocytes to test whether they are capable of undergoing ER stress response. It is only after we fully understand the physiological functions of cell organelles in

avian erythrocytes that we can tackle the question of how mammalian erythrocytes function without cell organelles.

Lastly, studies that compare erythrocyte traits between ectothermic and endothermic animals would be informative. Phylogenetic studies that incorporate all vertebrates should be employed to gain further insights into the evolutionary implications of erythrocyte enucleation (Supplementary figure S3), and whether evolution of endothermy plays a role in the evolution of erythrocyte enucleation in mammals. Specifically, phylogenetic comparisons between endotherms and ectotherms would provide information on whether the reported MCHC and MCV values in birds and mammals are restricted to endothermic lineages or not. Our preliminary analysis indicated that bony fishes exhibited higher MCHC than birds and mammals, but the significance was lost after correcting for phlogeny. It has been shown that a few species of attenuated or miniaturized salamander in the family Plethodontidae possess both nucleated and high levels of enucleated erythrocytes (> 80%) in circulation (67). The non-attenuate species also possess enucleated erythrocytes, but at much lower levels (< 10%)(67, 95). Interestingly, the enucleation of erythrocytes in the family Plethodontidae has been documented to evolve independently several times (95). However, it is important to note here that these enucleated erythrocytes in salamanders are unlike their mammalian counterparts since they often have irregular size and shape with non-mammalian cytoskeletal structure (13). Moreover, the presence of numerous free nuclei in the blood suggest that the enucleation might be the result of random cell breakage in circulating blood (95). That being said, the erythrocyte enucleation process and its adaptation in these salamanders are still important to investigate because they could potentially provide insights for enucleation in mammalian erythrocytes.

Perspective and Significance

Existing hypotheses regarding the evolution of enucleated erythrocytes in mammals fail to explain why and how birds retained cell organelles in their erythrocytes. It is possible that birds retain cell organelles in their erythrocytes whereas mammals extrude cell organelles from their erythrocytes as different mechanisms to achieve the same evolutionary outcomes while facing similar environmental and evolutionary selection pressure. In order to truly understand the evolutionary and physiological implications of enucleation, it would be valuable to first understand the physiological functions of various cell organelles in avian erythrocytes. We suggest taking an integrative and comparative approach, using tools from cell and molecular biology, organismal physiology, and evolutionary biology to revisit the century old idea of enucleated versus nucleated erythrocytes. This will encourage evolutionary biologists to rethink the evolutionary implications of enucleation, meanwhile inspire biomedical scientists to study various erythrocyte related diseases from an evolutionary perspective.

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Author contributions

KNY and YZ collected the data. KNY conducted the statistical analyses. KNY and YZ jointly conceived the study and wrote the manuscript. All authors gave final approval for publication.

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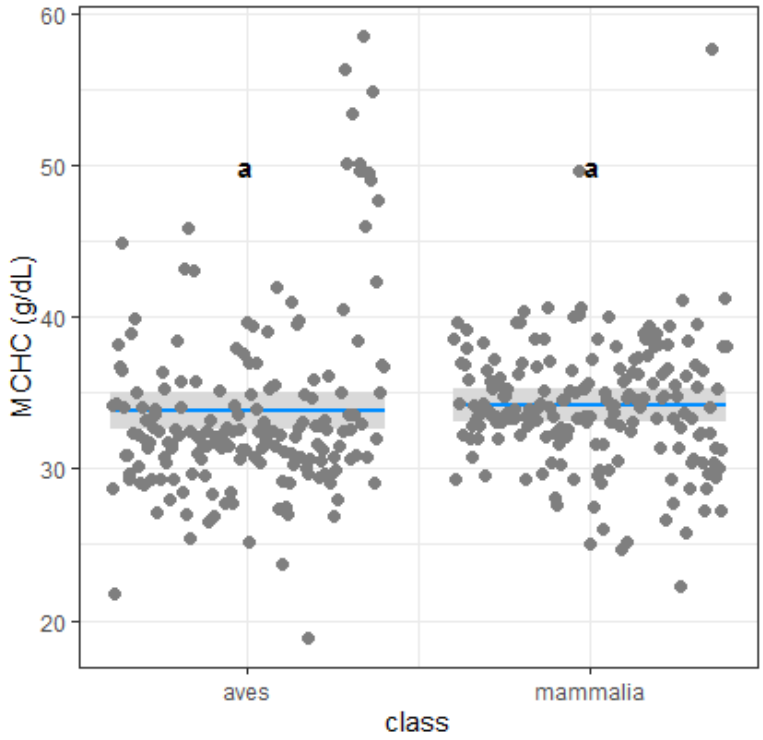
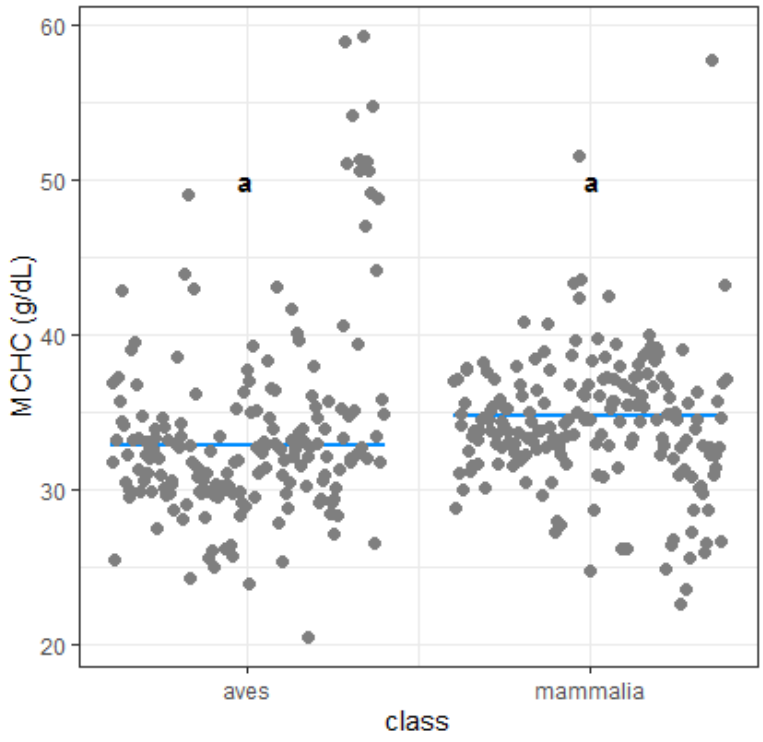
Figure 1. Relationship between A) Class and mean cell haemoglobin concentration (MCHC; OLS), B) Class and MCHC (PGLS), C) Class and mean cell volume (MCV; OLS), and D) Class and MCV (PGLS). Data shown individual species data and means generated based on either ordinary least squares or phylogenetic least squares. Different letters denote statistical significance. OLS: ordinary least squares; PGLS: phylogenetic generalized least squares.

Figure 1

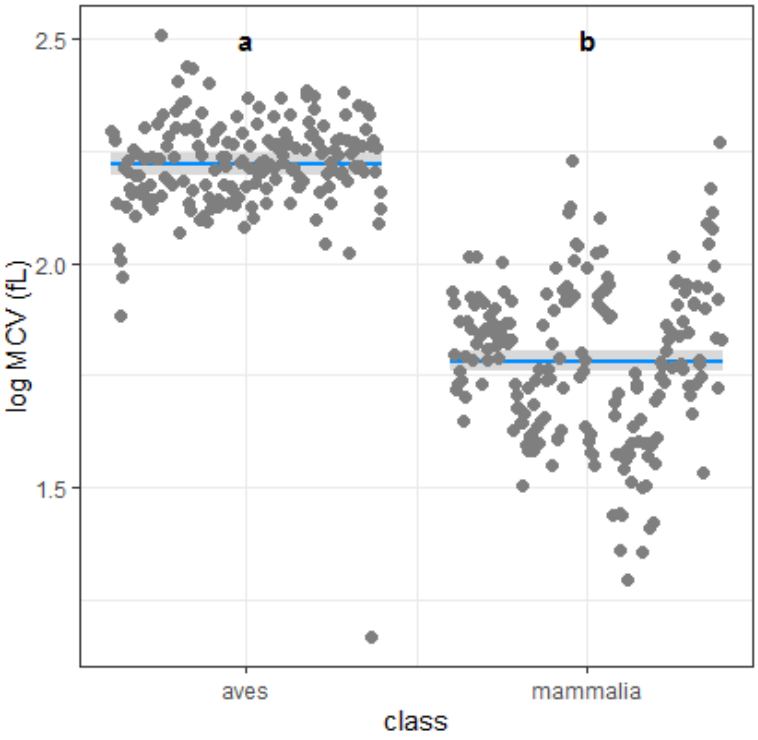
1A



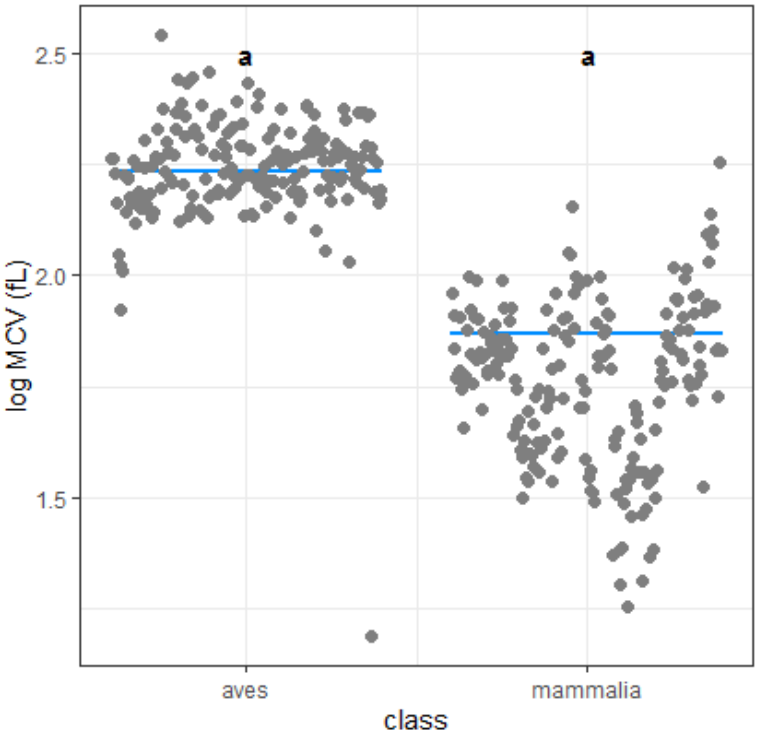
1B



1C


a
b
2.5
2.0
1.5
log MCV (fL)
aves
mammalia
class

1D


a
a
2.5
2.0
1.5
log MCV (fL)
aves
mammalia
class