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An investigation of factors influencing erythrocyte morphology of red-backed salamanders (*Plethodon cinereus*)

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Abstract

Amphibians have long been known to display wide variation in erythrocyte morphology across species, but within species there has been little attention given to individual variation in red blood cell morphology. We captured 49 red-backed salamanders (*Plethodon cinereus*) from central Pennsylvania, USA and used image analysis procedures to measure erythrocyte morphology (size and shape) on blood smears made from all individuals. We then statistically examined whether variation in snout-vent-length, sex, tail loss, or capture location influenced these cell variables. Only snout-vent-length affected erythrocyte size and shape, with increasing body sizes associated with increasing cell areas and increasingly rounder cells. Further, erythrocyte shape was also associated with a measure of body condition that was corrected for body size, such that individuals with high body condition scores had rounder cells. Given the oxygen-carrying role of erythrocytes in all vertebrates, we suspect this discovery is related to size-related changes in oxygen demand, since total oxygen consumption increases with body size in an allometric manner. While our results warrant further investigation to understand the mechanism, the association we found between cell roundness and both body size and condition nevertheless indicates this parameter could be used to assess the health state of plethodontid salamanders in future research, provided non-destructive sampling is employed. Our results also underscore the value of hematological investigations in the study of animal biology.

Keywords

Erythrocyte morphology; salamander; *Plethodon cinereus*; body size; image analysis

Introduction

In all vertebrates, erythrocytes are responsible for storing and transporting oxygen throughout the body, and are composed of little else but a nucleus (except in mammals) and hemoglobin in cytoplasm. There is considerable variation in cell morphology among species, especially within the amphibians (Vernberg, 1955; Kuramoto, 1981),

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and this variation is thought to stem from variation in metabolism (Smith, 1925; Vernberg, 1955). Until recently, little attention has been given to intraspecific variation in cell morphology. A recent study of erythrocyte dimensions in an aquatic salamander found that certain erythrocyte dimensions change with growth (Davis, 2008). In this case, increases in body size were accompanied by greater cell widths and shorter lengths in paedomorphic mole salamanders (*Ambystoma talpoideum*), which lead to larger individuals having more rounded-appearing cells. This study was made possible by the recent advances in image analysis technology (Davis et al., 2004; Davis, 2007; Davis and Grayson, 2007; Davis, 2008), which allows for rapid and objective measurement or quantification of macro- and microscopic features. Furthermore, this technology is ideal for investigations of subjects relevant to animal physiology such as of intraspecific variation in cell morphology, a subject in which little is known.

Plethodontid salamanders are an interesting study subject in terms of erythrocytes because they are lungless. Even though it is known that plethodontid salamanders consume nearly equal amounts of oxygen as lunged salamanders (Feder, 1976), the factors that allow for this in the absence of lungs are not known. While erythrocytes of most amphibians are nucleated, members of the plethodontid salamander family are known to have varying numbers of anucleated red blood cells (Villolobos et al., 1988), which were thought by some to be an adaptation to facilitate oxygen carrying (Turner, 1988). However, comparison of cells across 85 species of plethodontids and observations of free nuclei in blood have lead others to propose they result from simple cell breakage after traversing miniaturized capillaries, especially in small species (Villolobos et al., 1988). These authors further demonstrated that numbers of anucleated cells do not change with increasing body size, which if it did, would have indicated some oxygen-carrying role, given the known higher oxygen demands of larger individuals (Feder, 1976, 1977).

We captured and examined a set of red-backed salamanders (*Plethodon cinereus*) from two forested sites in central Pennsylvania, USA and conducted the present study of their erythrocytes to determine if and how erythrocyte morphology (size and shape) in this lungless plethodontid salamander vary with factors such as body size, sex, tail loss and capture location. We specifically used image analysis procedures to examine two components that explain most variation in cell size and shape - total area (i.e. the two-dimensional surface area of one flat side) and aspect ratio (L/W), which reflects the shape of the cell. We also asked if either of these parameters is associated with a common field measure of health in salamanders, their body condition, which we calculated as size-corrected mass.

Methods

Collecting and processing salamanders

As part of an unrelated study, 49 red-backed salamanders were hand-collected from sites within two deciduous forests in central Pennsylvania, USA on 4 October 2007; 25 from a mountainside in Hawk Mountain Sanctuary (HMS) and 24 from a valley in

Hopewell Furnace National Historic Park (HFP). All of the collections were taken from underneath untreated wooden coverboards and woody debris. After capture all individuals were placed in petri dishes and put on ice for transport to the lab, where they were held in the refrigerator for 7 days in individual petrie dishes. On the day of processing, each salamander was removed from its dish, blotted dry, weighed using an electronic balance and its snout-vent-length was measured to the nearest millimeter. We also recorded if the tail was intact (i.e. not automatized) and assigned each salamander to sex using external characteristics or from later dissection. Next, each was killed via overdose of MS-222 as specified under our university animal care protocol (# AUP2006-10041) which allowed for killing and collection of tissues from this species for the separate study. The animals were then decapitated and blood that welled from the heart region was blotted onto a microscope slide and a second slide was used to draw the blood into a standard blood film for microscopy. Slides were air-dried then later stained with giemsa.

Erythrocyte measurements

Slides were viewed with a light microscope under 1000X oil immersion, and erythrocytes were clearly identifiable at this magnification. We selected 10 fields of view for each salamander and obtained digital photographs of each field using a mounted Cannon digital camera. When all slides were processed we imported the digital images into Adobe Photoshop with FoveaPro plugins (Reindeer Graphics, Inc.) installed. The software was calibrated for actual dimensions using a digital photograph of a standard stage micrometer taken at 1000X. Thereafter, erythrocytes in the images were measured following image analysis procedures already established (Davis, 2008; Davis and Holcomb, 2008). In these procedures, cells in the field that are not touching other cells are automatically selected for measurement, and the user need only ensure immature cells and non-erythrocytes (i.e. leukocytes, thrombocytes) were not selected. The software then records the area (in μm^2), length and width (μm) and calculates the aspect ratio (L/W) of each selected cell. When all cell measurements were complete we calculated the average area and aspect ratio of erythrocytes for each salamander, which we used in our analyses.

Data analysis

We used ANCOVA to examine factors that may influence erythrocyte size and shape in this study. Thus, either erythrocyte area or aspect ratio were used as dependent variables, while categorical independent variables included sex, tail condition (intact or broken), and location (HMS or HFP), and snout-vent-length was included as a continuous covariate to examine the possible effect of body size. We next wished to determine if either cell area or aspect ratio relates to body condition of salamanders. We therefore obtained a measure of body condition by dividing mass by SVL for all salamanders that had intact tails. This variable is commonly used as a field measure of body condition in salamanders (Karraker and Welsh, 2006). This variable was then compared to both erythrocyte parameters using simple linear regression. All tests in this study were performed using Statistica 6.1 software (Statistica, 2003).

Results

General results

We collected 49 salamanders (males: n = 24; females: n = 25), which ranged from 36-50 mm in snout-vent length, and this distribution was approximately normal, though there were only 3 individuals at the low end (i.e. less than 37 mm). Because these few individuals could influence the outcome of statistical analyses in this study, we conducted each analysis involving body size with and without these individuals. In this species, individuals above 32 mm for males and 34 mm for females are considered adults (Petranka, 1998), so we therefore can consider all individuals here to be adults. Of these, 24 individuals (49%) were males and 25 (51%) were females. Moreover, 35 individuals had intact tails while in 14 the tail had been broken (prior to capture). From all individuals a total of 3,602 erythrocytes were measured using image analysis. The number of cells measured per individual ranged due to variations in smear density and therefore the number of cells that could be isolated by the image analysis program, but at least 35 cells per individual were measured in all cases (range=35-102, mean=74). For comparison with other studies, the average erythrocyte dimensions of all individuals are shown in table 1. Values of mean length and width were 27.6μm and 13.8μm, respectively, which is comparable to values of 30.6μm and 16.3μm obtained by previous authors (Vernberg, 1955).

ANCOVA results

Of all variables considered, body size (measured as SVL) was the only factor that significantly affected both erythrocyte size and shape (table 2). In the analysis of erythrocyte area, we found no support for any of the two-way interaction terms and they were therefore removed. Results from simplified model with only main effects revealed no significant effects of sex, tail or capture location (table 2), but a significant effect of snout-vent-length. This relationship was positive so that larger salamanders tended to have larger cells (fig. 1A). Furthermore, repeating this analysis without the three smallest salamanders yielded the same results, in that there was a significant (positive) effect of SVL ($F_{1,41}$=6.62, p=0.014). For the analysis of erythrocyte shape (aspect ratio), we again found no support for any two-way interaction term and again in a simplified model the only significant effect was SVL (table 2), such that larger salamanders tended to have erythrocytes with smaller aspect ratios (i.e. were more round; fig. 1B). Repeating

Table 1.
Average dimensions of erythrocytes from all 49 red-backed salamanders examined in this study, as measured using image analysis (see methods)

Cell Parameter	Mean	Std.Dev.	Minimum	Maximum
Area (μm^2)	304.6	17.0	267.9	338.7
Length (μm)	27.6	1.2	24.8	30.1
Breadth (μm)	13.8	0.8	12.3	15.8
Aspect Ratio	2.0	0.2	1.7	2.4

Table 2.
Results of ANCOVA models examining factors influencing erythrocyte size (area) and shape (aspect ratio) in red-backed salamanders. All two-way interaction terms were initially included in both models but all were removed when found not significant. Tail was included as a dichotomous variable (intact or broken)

Independent	Dependent	df	MS	F	p
Erythrocyte Area	Location	1	458.60	1.78	0.189
	Tail	1	9.08	0.04	0.852
	Sex	1	0.06	0.00	0.988
	SVL	1	1120.78	4.34	0.043
	Error	44	258.07		
Erythrocyte Aspect Ratio	Location	1	0.00	0.02	0.895
	Tail	1	0.00	0.06	0.808
	Sex	1	0.03	1.01	0.320
	SVL	1	0.20	7.01	0.011
	Error	44	0.03		

this analysis without the three smallest salamanders also indicated a significant effect of SVL ($F_{1,41}$=6.50, p=0.015).

Observations of cells

To visualize the effect of salamander body size on cell dimensions, photomicrographs of erythrocytes of a small (SVL=36mm) and large (SVL=50mm) salamander are presented, as seen under 1000X oil immersion (fig. 2A,B). In the small salamanders the cells appeared elongated or elliptical, often with eccentric nuclei. Moreover, misshapen

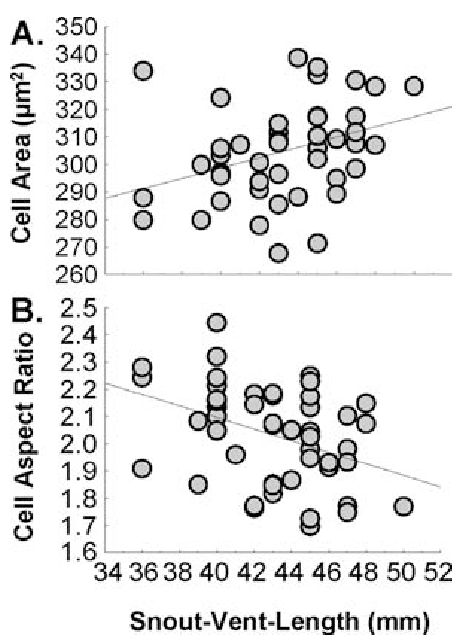

Figure 1. Relationships between red-backed salamander body size (snout-vent-length) and erythrocyte size (2-dimensional surface area; A), and shape (aspect ratio, or length/width; B).

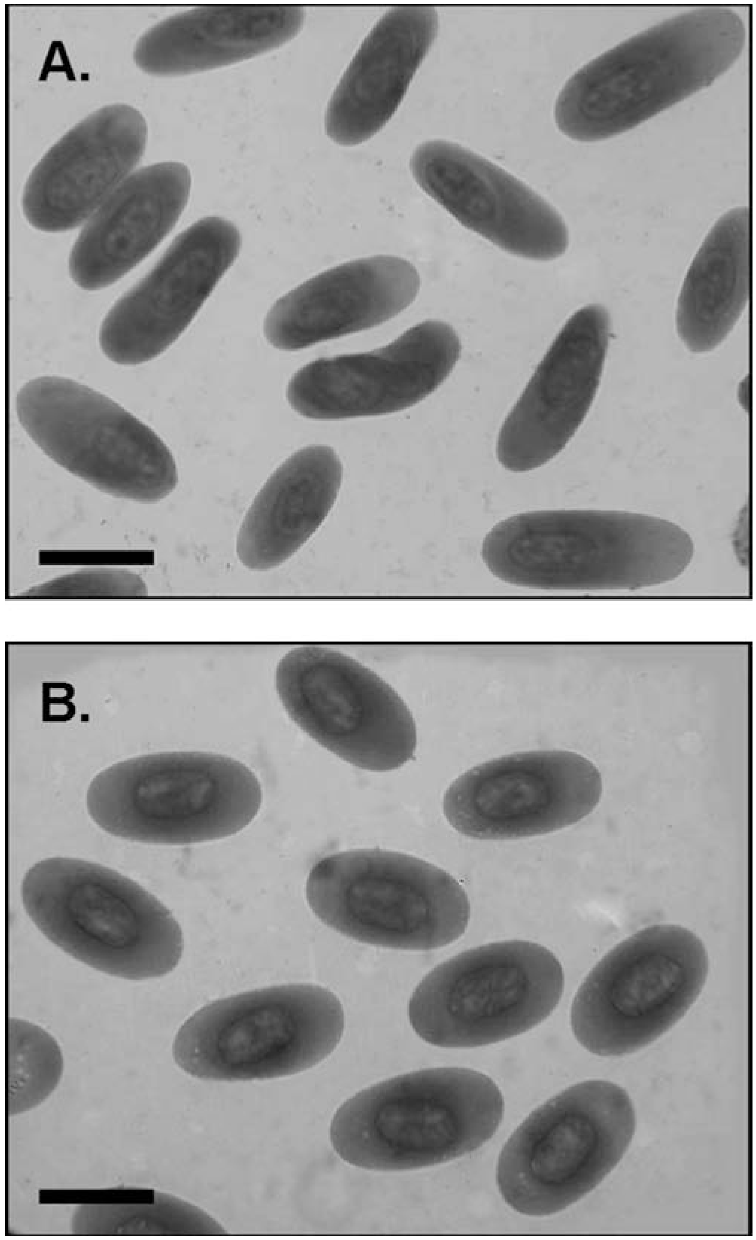

Figure 2. Photomicrographs of erythrocytes, as seen under 1000x oil immersion, from two red-backed salamanders: one small (SVL=36mm, A) and one large (SVL=50mm, B). Scale bar represents 20 μm.

cells were occasionally observed (fig. 2A). In contrast, cells of large salamanders tended to be wider and therefore appear rounded, with centrally-placed nuclei (fig. 2B).

Erythrocyte morphology and body condition

Of the 35 individuals with intact tails, body condition scores calculated from mass and SVL were approximately normally-distributed. There was no significant linear relationship between erythrocyte area and these body condition scores ($F_{1,33}$=0.17, p=0.682). However, there was a significant relationship between body condition and cell aspect ratio ($F_{1,33}$=11.32, p=0.002) such that as body condition increased in

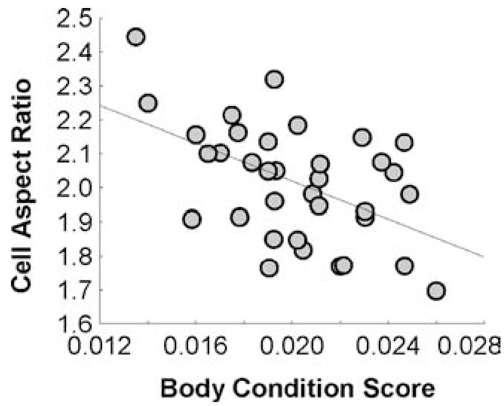

Figure 3. Relationship between size-corrected body condition (mass divided by SVL) and erythrocyte shape (aspect ratio) for all red-backed salamanders with intact tails in this study (n=35). Smaller aspect ratios indicate more rounded cells; larger body condition scores indicate more robust salamanders.

salamanders, erythrocytes became rounder (fig. 3). As in the analyses of body size, here there were two individuals at the extreme low end of body condition, which could be driving this relationship, so we therefore repeated this analysis without these salamanders. Doing so did not change the outcome of the results, in that there was still a significant linear relationship ($F_{1,31}$=4.20, p=0.049).

Discussion

This investigation uncovered a close association between body size, body condition and erythrocyte morphology in red-backed salamanders. Specifically, in both body size and condition, red blood cell roundness appeared to have diagnostic value for predicting these two measures of fitness. This result is somewhat consistent with prior findings of ontogenetic changes in erythrocyte roundness in an aquatic salamander (Davis, 2008), although in that study, adults and juveniles were examined while only adults were studied here. This consistency though, especially in both an aquatic and terrestrial salamander, speaks to the possibility of this trend being present in other salamanders.

We suspect the reason for the variation in cell size and roundness is related to variations in hemoglobin content within cells. Indeed, cells in small salamanders had the appearance of thin, unfilled 'bags', especially since the nuclei appeared randomly-placed (as if floating freely), and cells often were slightly misshapen (fig. 2A), while those of larger individuals appeared 'filled', with nuclei nearly always in the center, and rarely were these cells misshapen (fig. 2B). Moreover, in these cells the rounded shape appeared to result from greater expansion of the sides than the lengths. This is confirmed by other data on cell dimensions collected during this study (but not specifically analyzed). There was a positive correlation between erythrocyte width and SVL (r=0.47, p=0.001). Meanwhile there was no relationship between cell length and SVL (r=-0.13, p=0.373), which suggests that cells remain the same length throughout life in red-backed salamanders. However, they appear capable of expanding their widths, leading to the rounded appearance. Because erythrocytes are composed of little else but hemoglobin (Thrall et al., 2004), the observed variation in cell dimensions is likely associated with increases in hemoglobin content, although direct measurement of hemoglobin would be needed to confirm this. However the relationship we found between salamander body condition and cell roundness supports this idea.

Increasing the amount of hemoglobin per cell would be one way to meet the body's increasing demands for oxygen as salamanders grow in size (Feder, 1976, 1977). Energetically, this would be a conservative strategy as well, given the high amounts of energy required to create and maintain cell membranes (Szarski, 1983). The possibility exists however, that the size increases in erythrocytes we observed merely reflect allometric scaling of body and cells (i.e. as capillary size grows, cell sizes increase). This idea has been suggested in comparison of erythrocyte sizes and capillary dimensions across vertebrates (Snyder and Sheafor, 1999). Indeed, the highly elongated cells of small salamanders we examined could indicate some adaptation for traversing thin capillaries. However, this idea seems unlikely given the apparent irregularities in cell shape and nuclei placement of small salamanders (fig. 2A).

Finally, while this discovery of ontogenetic and condition-related variation in erythrocyte morphology clearly warrants further study to clarify the physiological mechanism, the existence of the phenomenon could nonetheless serve as a useful diagnostic parameter for aiding the assessment of health status in plethodontid salamanders, either in live specimens using non-destructive sampling or in euthenized specimens, as was done here. This could also be used in combination with other hematological parameters such as leukocyte profiles, which are being increasingly adapted to amphibians to assess physiological stress levels (Davis and Maerz, 2008a, 2008b). Indeed, this approach (cell morphology) is already used in veterinary clinics, where animals with smaller than normal erythrocytes (indicating low hemoglobin) are diagnosed as anemic (Thrall et al., 2004). By this same logic, the opposite should therefore be true. In support of this, large erythrocyte size (which indicate high hemoglobin content) has been shown to correspond with several measures of fitness in adult birds (Bearhop et al., 1999). Erythrocyte size (indexed by mean corpuscular volume) was found to be the most important predictor of nestling survival of multiple blood parameters measured (Nadolski et al., 2006) and Banbura et al. (2007) showed that direct measures of hemoglobin content of nestlings predict their survival. Our results using a computer-assisted measure of erythrocyte size in an amphibian are consistent with these, in that they also point to an association between erythrocyte size and measures of fitness, but more generally they highlight the value of hematological investigations in the study of animal biology.

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