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ORIGINAL ARTICLE

Ontogenetic changes in erythrocyte morphology in larval mole salamanders, *Ambystoma talpoideum*, measured with image analysis

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Abstract Knowledge of the erythrocyte morphology in an animal can provide information regarding its genome size, metabolic rate, exposure to pollutants and acclimatization to its environment. In amphibians, there are thought to be two morphological forms of erythrocytes, a larger, elongated larval type and a smaller, rounder adult form. I examined erythrocyte morphology in larval and paedomorphic adult mole salamanders, *Ambystoma talpoideum* using an image analysis approach to measure dimensions of 30,213 erythrocytes in 66 individuals captured from a single pond. The erythrocytes of this species averaged 590.3 μm^2 in area, 34.7 μm in length, and 22.6 μm in width, which is comparable to values reported from related Ambystomatid species. There was a tendency for cells to decrease in length with increasing salamander size, and a stronger positive relationship between body size and erythrocyte width, so that cells were more rounded overall in larger individuals. There was also less variability in certain erythrocyte dimensions in larger individuals. However, cells did not decrease in area as salamanders grow, contrary to expectations. I conclude with comments on the image analysis approach used in this study, which proved especially useful in gathering this data, as it allowed for large sample sizes, direct measurement of surface areas (of one side of cells), and was nearly fully automated.

Keywords Erythrocyte morphology · Mole salamanders · *Ambystoma talpoideum* · Image analysis

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Introduction

The measurement of erythrocyte dimensions is often an important component to standard hematologic surveys of novel species (Hartman and Lessler 1963), comparisons across species (Atatür et al. 1999), and studies of environmental, seasonal, or altitudinal acclimatization (e.g., Ruiz et al. 1989; Pagés et al. 1992; Ruiz et al. 2004). Measuring erythrocyte size can also provide information regarding the genome size of a species (Kuramoto 1981; Gregory 2001). In amphibians, erythrocyte size has long been known to correlate negatively with metabolic rates, both at the organism level (Smith 1925; Vernberg 1955) and the tissue level (Monnickendam and Balls 1973). This relationship stems from the fact that larger surface-area-to-volume ratios in smaller cells allow for more efficient exchange of oxygen. This idea is exemplified in intraspecific comparisons of amphibians at different altitudes, where animals at higher latitudes have smaller erythrocytes (Ruiz et al. 1983, 1989), presumably to maximize cellular efficiency of oxygen transport and exchange in a low-oxygen environment. Moreover, measurements of erythrocyte size can also be used as a diagnostic assay to assess the effects of air pollution in animals (Llacuna et al. 1996). Clearly, knowledge of the erythrocyte morphology of an animal has many applications; however, one issue that has not been well studied is the relationship between ontogenetic growth and erythrocyte morphology, especially in animals such as amphibians that have indeterminate growth.

In larval amphibians, there are thought to be two general types of erythrocytes, a larval and adult form, which are differentiated by size and morphology (e.g., Hollyfield 1966; Benbassat 1970; Broyles et al. 1981). The larval form is large and elongated while the adult form is smaller and rounder, with adult erythrocytes more uniform in size and

shape than in larvae (Benbassat 1970). The transitions from larval to adult cells begin at the onset of metamorphosis (Hollyfield 1966; Hasebe et al. 1999). Furthermore, Hollyfield (1966) found that the proportion of larval cell forms decreases from approximately 75% of the red cell population to zero (i.e., all adult forms) in a span of 12 days after the onset of metamorphosis.

Erythrocytes have traditionally been measured manually under light microscopes and using an ocular micrometer (e.g., Monnickendam and Balls 1973; Kuramoto 1981; Ruiz et al. 1983; Atatür et al. 1999), or manually measured from photographs of smears (e.g., Hollyfield 1966; Broyles et al. 1981), both of which can be time consuming. Recent advances in computer-assisted image analysis techniques now allow for more rapid and detailed enumeration and measurement of microscopic objects (Davis 2007; Davis et al. 2004), and this approach can easily be adapted to the measurement of erythrocytes.

In this study, I examined blood smears from a set of larval and adult paedomorphic mole salamanders, *Ambystoma talpoideum*, and used image analysis techniques to measure their erythrocyte dimensions, with the intent to examine how erythrocyte morphology varies with salamander size. As this was the first study to examine erythrocyte morphology in *A. talpoideum*, a secondary goal was to establish reference values for the species. Given the prior observations of larval vs adult cells in amphibians, I expected to find smaller cells in general in larger individuals. I also looked for evidence of less variable cell dimensions in larger individuals. Finally, I highlight the advantages of the image analysis method for measuring erythrocyte dimensions, which include more accurate measurements, greater sample sizes, and require less time to obtain than previously considered in hematological work.

Materials and methods

Capturing and processing salamanders

Between 13 and 15 June, 2006, I dipnetted 66 nonbreeding paedomorphic salamanders from a single permanent pond near Athens, Georgia, USA. The collected salamanders ranged in size (see results) and therefore represented a set of varying-aged individuals. All individuals were placed in a clean plastic container of pond water and transported immediately to the lab. Within the same day of capture, each salamander was killed via immersion in MS-222, and its body length (from nose to tail tip) was measured. The animal was decapitated and a blood sample was collected from the cardiac region with a heparinized capillary tube. A drop of this blood was placed on a clean microscope slide, and a second slide was used to smear the blood. Blood smears were air-dried then stained with Giemsa (Fig. 1).

Processing blood smears

Blood smears were examined (at 200×) using a light microscope fitted with an 8 megapixel digital camera mounted on a trinocular head, and for each smear five fields of view were photographed. Fields were randomly chosen but fields with low numbers of cells were not selected. When all smears had been examined, the digital images were imported into Adobe Photoshop with Fovea Pro (Reindeer Graphics) plugins installed. An image of a stage micrometer (photographed with the same setup) was used to calibrate the actual dimensions of the blood smear images. Then a series of image analysis routines were created to (1) automatically select the erythrocytes in each image, and (2) measure their dimensions (Fig. 2). In step 1, only erythrocytes that were not overlapping and that were intact were selected. In step 2, the measured variables included surface area (i.e. the area of one flat side of the cell, in square micrometer), length (i.e. the longest possible line within each cell, in micrometer), breadth (line perpendicular to the length, in micrometer), and these data were automatically saved in a separate text file.

Data analysis

For each salamander, I calculated the average of each erythrocyte variable (area, length, width) as well as the standard deviations of each variable. Pearson correlations were then used to examine relationships between salamander body size (body length) and the average erythrocyte

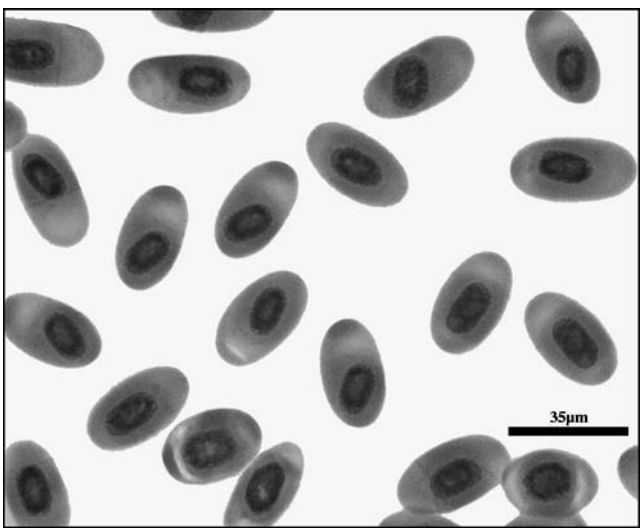

Fig. 1 Photomicrograph of typical erythrocytes in a blood smear (at 1,000×) from an adult-sized paedomorphic mole salamander

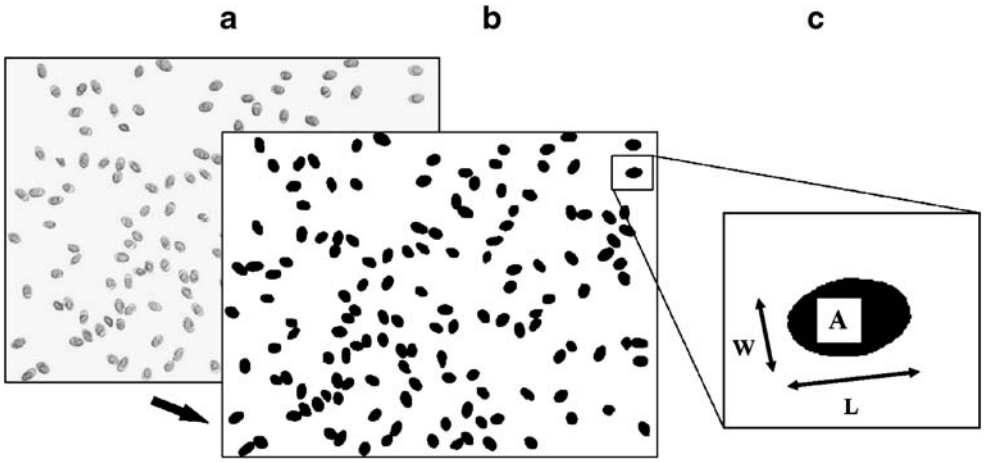

Fig. 2 Summary of blood smear image analysis procedures. Erythrocytes in initial images (**a**) were selected based on degree of isolation and intactness (**b**), then a measurement routine was initiated to automatically measure length, width and area of each erythrocyte (**c**), based on a known pixel to micrometer ratio calculated previously with a stage micrometer. A total of 139 erythrocytes were measured in this image. Five such images per salamander were examined

length, breadth and area. I also examined the possible relationships between body size and the variability of erythrocyte dimensions, using the standard deviation of erythrocyte area, length and width for each salamander. Analyses were performed using Statistica software (Statistica 2003).

Results

Salamander size

In the set of 66 salamanders captured, there was an approximate normal distribution of body sizes (Fig. 3). The mean body length measurement was 71.0 mm (min: 60.3, max: 81.9). The lack of a clear morphological difference between larval forms and nonbreeding paedomorphic adults made it difficult to separate the individuals into either form. However, those individuals that approached 80 mm were most likely paedomorphic adults, since prior study of salamanders from the same pond showed that nearly all breeding (paedomorphic) adult mole salamanders in this population were over 80 mm long (unpublished data). There were at least eight such individuals (12%) that were over 78 mm in length in the current data set. Furthermore, I detected no external abnormalities or outward signs of disease in any of the salamanders examined in this study.

Mole salamander erythrocyte dimensions

A total of 30,213 erythrocytes were measured across all 66 salamanders in this study. Within individuals, the number of erythrocytes measured varied (between 100 and 400) because of variations in smear density, or the erythrocyte selection criteria. The overall average erythrocyte area measurement was 590.3 μm^2 and ranged from 310.2 to 1071.0 μm^2. The distribution of area values was a normal, bell-shaped curve (Fig. 4). Meanwhile, the average length of *A. talpoideum* erythrocytes was 34.7 μm (min: 26.6,

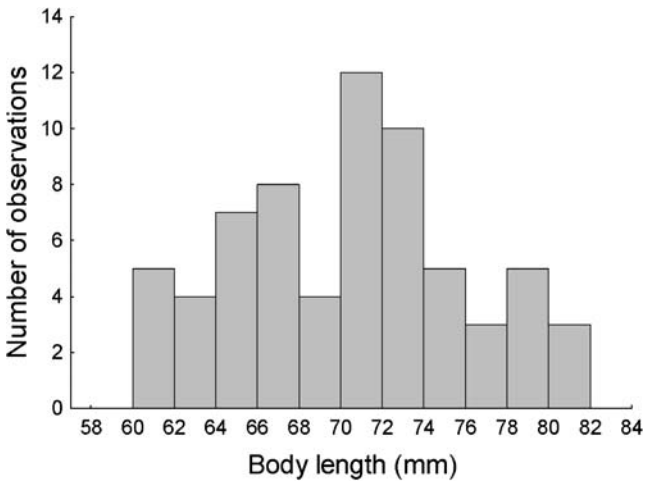

Fig. 3 Range of body sizes of all 66 salamanders captured for this study

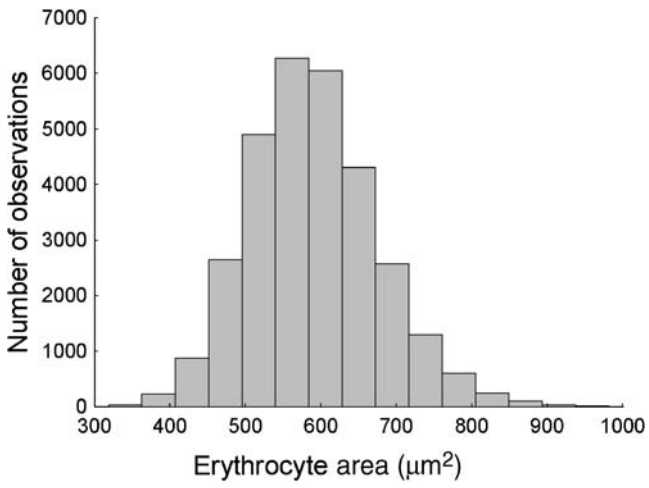

Fig. 4 Frequency distribution of erythrocyte area measurements (i.e. area of one cell side, in square micrometer) obtained from all 66 salamanders in this study

max: 44.4) and the average width was 22.6 μm (min: 14.9, max: 35.2).

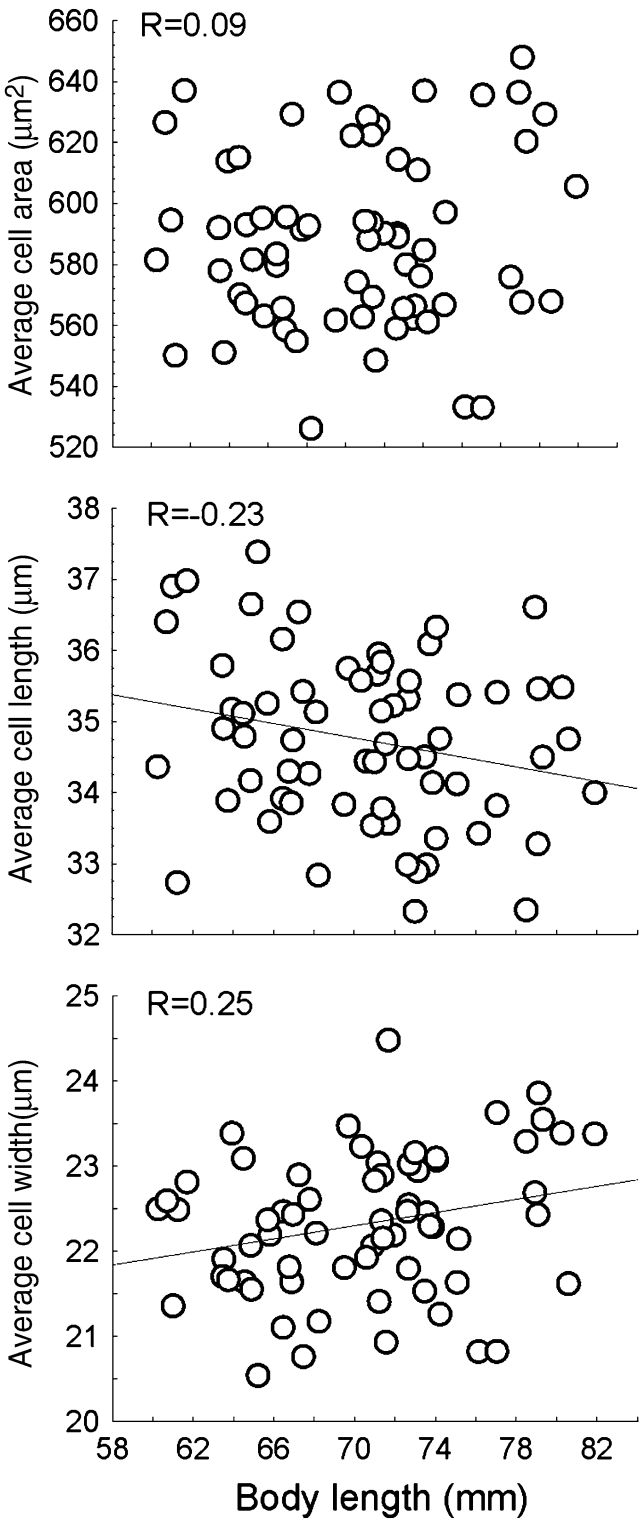

Fig. 5 Relationships between salamander body size and erythrocyte dimensions. For each salamander, the average value of its erythrocyte area (*top graph*), length (*middle*), and width (*bottom*) is shown

Ontogenetic effects

There was no significant relationship between salamander body size and erythrocyte area (r=0.09, p=0.461; Fig. 5). There was a tendency for erythrocytes to decrease in length with increasing salamander size (r=−0.23, p=0.062). Conversely, there was a significant positive relationship between salamander size and average erythrocyte width (r=0.25, p=0.042). Thus, in the salamanders examined in this study, erythrocytes did not show evidence of becoming smaller with increasing body size, but in fact became rounder (i.e. smaller lengths, larger widths). Regarding cell variability within individuals, there was no relationship between body size and the standard deviation of erythrocyte area (r=−0.19, p=0.132), but there were negative relationships between

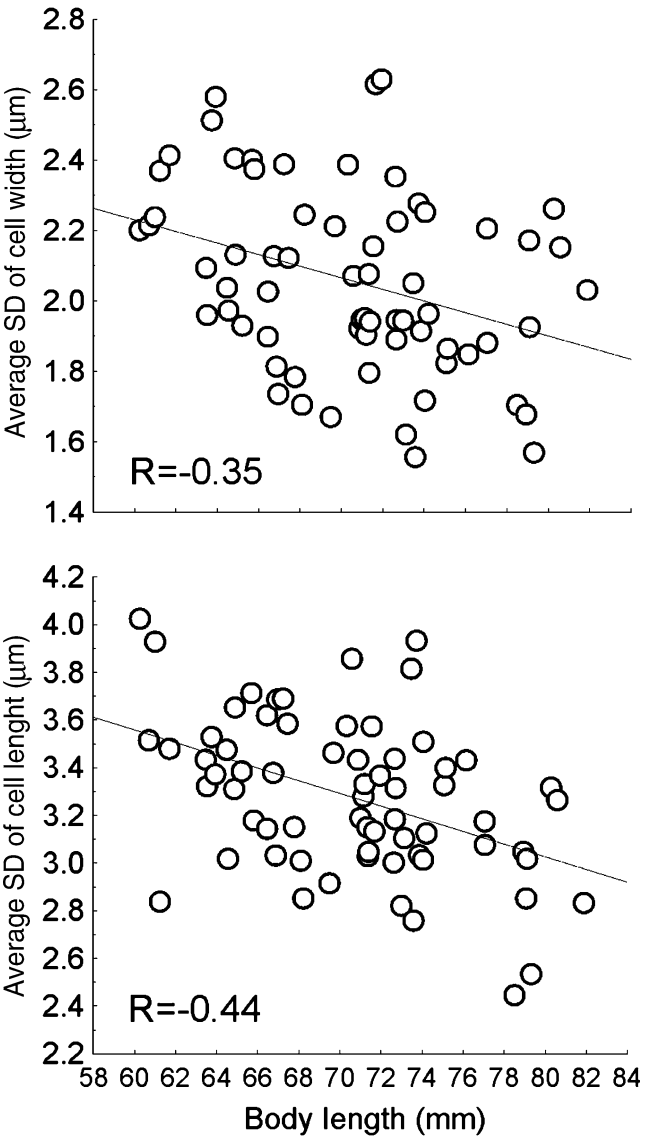

Fig. 6 Relationships between salamander body size and variability (i.e. standard deviation of values for each individual) in erythrocyte widths (*top graph*) and lengths (*bottom graph*)

body size and the standard deviation of cell length (r=−0.44, p<0.001) and cell width (r=−0.35, p=0.005; Fig. 6). In other words, erythrocytes become less variable in length and width as body size increases in mole salamanders.

Discussion

This study is the first to report erythrocyte dimensions of *A. talpoideum*. In related *A. tigrinum*, average erythrocyte length is 34.5 μm and width is 19.0 μm, while in *A. maculatum* the erythrocytes are 37.9 μm long and 23.9 μm wide (Vernberg 1955). These are comparable to the length and width values obtained here of 34.7 and 22.6 μm. Importantly though, certain dimensions appear to vary with salamander growth. As might be expected based on the reported differences between larval and adult erythrocytes in amphibians, I found that as larval salamanders grow their erythrocytes become less elliptical and more round. Also as expected, erythrocytes become less variable in length and width as salamanders grow.

It is surprising to note that I found no evidence for decreasing erythrocyte size (i.e. area) as individuals grow, which is contrary to expectations based on prior observations by many researchers (Hollyfield 1966; Benbassat 1970; Hasebe et al. 1999). I also found no evidence of a bimodal distribution of cell sizes (Fig. 4), which has been observed in bullfrog tadpoles and signifies larval and adult erythrocytes (Broyles et al. 1981). One explanation for this discrepancy is that the majority of the prior observations have been on anurans, while to date erythrocytes of only one salamander species, *Hynobius retardatus*, have been examined (Wakahara and Yamaguchi 2001), and these researchers themselves could not state that the erythrocytes in this species typified those of all urodeles. It may be that not all conclusions based on anuran erythrocyte morphology can be used to predict urodele cell morphology. Indeed, there is other evidence that erythrocytes of urodeles are morphologically different from that of anurans, being larger and more elongated (Kuramoto 1981).

Comments on image analysis

The novel image analysis procedure used in this study to measure erythrocytes was highly useful for this purpose. To begin, this approach allowed me to measure 30,213 cells from the animals in this study, which is an order of magnitude more than prior studies have examined. Furthermore, this approach provided a direct quantification of each erythrocyte's surface area (albeit two-dimensional), which has traditionally been estimated from length and width measurements (e.g., Smith 1925; Vernberg 1955; Hollyfield 1966; Sinha 1983; Atatür et al. 1999). In addition to the breadth of data, this method was remarkably efficient. The entire measurement routine was automated so that the user only needed to ensure that damaged erythrocytes, leukocytes, and overlapping cells were not selected. This automation therefore made it possible for all 30,213 erythrocytes to be measured for this study within one afternoon, including the time needed to take the pictures of blood smears. This efficiency is not unlike that found when measuring images of parasite spores (Davis et al. 2004) and hemocytes in insects (Davis 2007) using variations of this method. Finally, not only did this allow a large number of cells to be measured, it was perhaps more objective than manual measurements, where cells are usually selected by the observer for measurement.

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