Research Paper

Morphological and Morphometric Variations of Erythrocytes in Clarias Gariepinus, *Bufo Bufo*, and *Agama Agama* Using Some Histological Stains

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ABSTRACT

Introduction: Erythrocytes are highly specialized and the most abundant cell type in vertebrates' blood. Their primary function is the transportation of oxygen to tissues of the body via hemoglobin.

Methods: The study was aimed at evaluating erythrocyte morphology in *Clarias gariepinus* (*C. gariepinus)*, *Bufo bufo* (*B. bufo*), and *Agama agama* (*A. agama*) using different stains; Also, morphometric analysis of erythrocytes was evaluated in stains with the best affinity. The blood samples of *C. gariepinus*, *B. bufo*, and *A. agama* were collected using a syringe. Smears from each animal were made on glass slides and stained with Giemsa, Hematoxylin and eosin (H&E), and methylene blue. Erythrocytes diameter was measured using a standardized ocular micrometer. The area and volume of the erythrocytes were calculated.

Results: Erythrocyte's shapes range from spherical in *C. gariepinus* to oval in *A. agama*. Giemsa demonstrated *C. gariepinus* and *B. bufo* erythrocytes better than H&E and methylene blue stains, while H&E demonstrated erythrocytes of *A. agama* better than Giemsa and methylene blue. Erythrocyte's diameter, area, and volume of *B. bufo* were significantly higher ($P<0.001$) when compared with *C. gariepinus* and *A. agama.*

Conclusion: Erythrocyte's shape, size, area, and volume vary in different species and could be used to study evolutionary trends. The variation in erythrocytes size is associated with chromosome number and genome size.

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1. Introductio

Erythrocytes are highly specialized and the most abundant cell type in vertebrates' blood. Their primary function is the transportation of oxygen to tissues of the body via hemoglobin [1, 2]. Except for ice fishes, all vertebrates have hemoglobin packed in their erythrocytes. Erythrocytes are used in the eco-physiological and eco-morphological evaluation of species [3, 4]. Blood cell parameters are used to evaluate the physiological and health status of individuals of the same specie [5]. Blood parameters such as volume, fragility, hematocrit, pH, number, and sizes may vary in different species and even among the same species for some reasons [6]. Factors that affect blood parameters in the same species include differences in altitude, body mass, age, sex, and diseases such as sickle cell and megaloblastic anemia [7, 8]. The variation in the blood parameters of different species results from divergent evolutionary changes [9].

Stains are used in histology to highlight tissue characteristics and to enhance contrast; they are employed based on their affinity to different tissues [10, 11]. Staining is a procedure undertaken in histological technique for studying tissue with a microscope; it involves introducing dyes into tissues for better visualization [12]. Staining has various applications in biological and medical sciences, including demonstrating certain substances within tissues and diagnosing different diseases [13]. The most commonly used histological stains include hematoxylin, eosin, methylene blue, cresyl violet, Giemsa, periodic acid Schiff (PAS), Leishman, and Papanicalau [14-16].

This study aimed to evaluate the affinity of different histological stains; Giemsa, Hematoxylin and eosin (H&E), and methylene blue on the erythrocytes of *Clarias gariepinus* (Catfish), *Bufo bufo* (Toad) and *Agama agama* (Lizard). Additionally, the erythrocytes morphology of stains with the best affinity were described and schematically drawn besides their morphometric analysis.

2. Materials and Methods

Experimental animal

Three of the following vertebrates were used for the study (*C. gariepinus*, *B. bufo*, and *A. agama*). *C. gariepinus* and *B. bufo* were obtained from Lake Alau in Maiduguri, Nigeria, while the *A. agama* was obtained from the University of Maiduguri campus. The research was approved by the Department of Human Anatomy ethical committee (Code: UM/HA/UGP19.20-095) and conducted following the National Research Council Guide for the use of laboratory animals (8th edition).

Smear preparation and staining

The blood sample of each animal was collected using a syringe. At least 10 smears from each animal were made on glass slides at different intervals and stained with hematoxylin ($C_{16}H_{14}O_6$) and eosin ($C_{20}H_6Br_4Na_2O_5$), Giemsa stain (mixture of buffered Azure B, methylene blue and eosin Y in methanol and glycerol) and methylene blue ($C_{16}H_{18}ClN_3S$). The stains were left for 5-10 minutes each, washed off under running water, and then the slides were observed under a light microscope (Leica Biosystems RM2235, Germany). Photomicrographs were taken at x400 magnification (40 objectives x10 oculars) using a microscope camera (AmScope, M500, USA).

Morphometric study

The diameters of the erythrocytes were measured using a standard ocular micrometer from the stains that produced the best contrast. The erythrocyte's diameter of an oval cell is the length of the short axis (Figure 1A), while the diameter of a spherical cell is the length of a straight line passing through the center of the cell, whose endpoints lie on the cell membrane (Figure 1B). The area and volume of each cell were calculated using the following Equation:

$$Area\ of\ spherical,\ area\ of\ oval\ cell=\pi ab,\ the\ volume\ of\ spherical\ cell=(4/3\pi)r^3,\ the\ volume\ of\ oval\ cell=(4/3\pi)a^2b$$

, where r is the radius, r=(diameter/2), a is the radius of the long axis, a=(long axis/2), b is the radius of the short axis, b=(short axis/2) (Figure 1C), π=3.142.

Statistical analysis

Data were analyzed with GraphPad Prism 7 (GraphPad Software, California, USA). A schematic diagram of best-stained cells was drawn. One-way analysis of variance followed by the Bonferroni post-hoc test was used to compare the erythrocytes' diameter of different vertebrates, and values were expressed as Mean±SEM. $P<0.05$ was considered statistically significant.

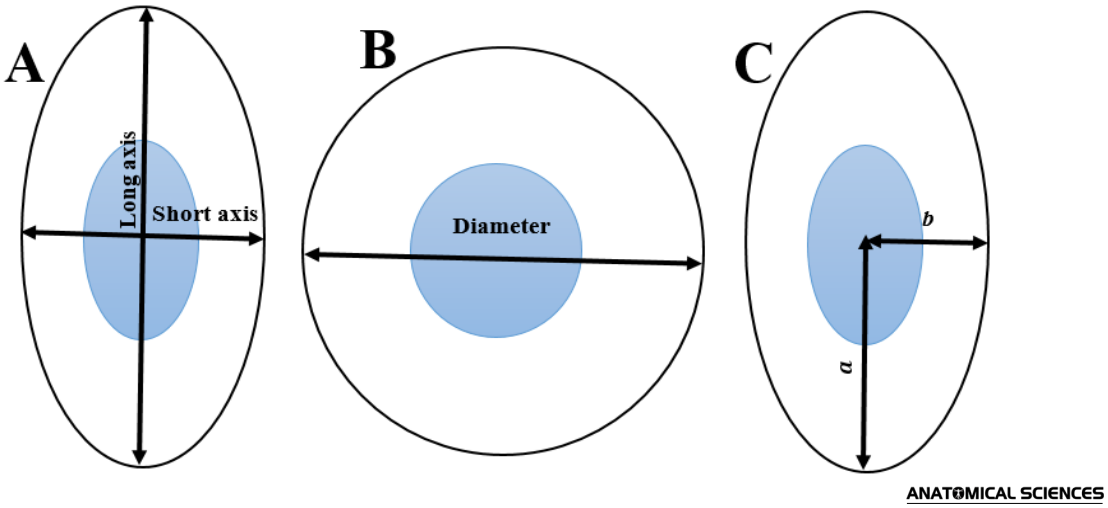

Figure 1. Measurement of erythrocytes' parameters, a=radius of the long axis and b=radius of the short axis.

3. Results

Morphology

The erythrocytes of *C. gariepinus* are spherical, as seen on Giemsa, H&E, and methylene blue stained slides. The nuclei are stained violet with Giemsa, pink with H&E, and blue with methylene blue (Figures 2-4). The cell membranes were distinguishable from the background in all stains but distinct in Giemsa stained cells compared to H&E and methylene blue stained cells (Figures 2-4). The cytoplasm showed less affinity for all the stains compared to their nuclei. The cytoplasm of erythrocytes in *C. gariepinus* had a higher affinity for Giemsa stain when compared to H&E and methylene blue (Figures 2-4).

Erythrocytes of *B. bufo* range from spherical to oval shapes, as demonstrated by Giemsa, H&E, and methylene blue stains (Figures 5-7). With Giemsa, the nuclei appeared dark blue with orchid cytoplasm, H&E stain highlighted blue nuclei with pink cytoplasm, while methylene blue stained cells showed a cytoplasm that was lightly stained blue with dark nuclei (Figures 5-7). Giemsa demonstrated *B. bufo* erythrocytes better than H&E and methylene blue stains.

The erythrocytes of *A. agama* are oval-shaped with purple nuclei and pink cytoplasm when stained with H&E (Figure 8). Giemsa and methylene blue stained cells showed no contrast between the nucleus and cytoplasm. The whole cell appeared blue in both Giemsa and methylene blue-stained cells (Figures 9, 10). H&E stain demonstrated the erythrocytes of *A. agama* better when compared to Giemsa and methylene blue stains.

Morphometry

The erythrocyte's diameter in *C. gariepinus*, *B. bufo*, and *A. agama* was 6.75±0.16 µm, 11.85±0.38 µm, and 7.10±0.26 µm, respectively. The diameter of *B. bufo* erythrocytes was significantly wider (P<0.001) compared to *C. gariepinus*; likewise, the *B. bufo* erythrocytes' diameter was significantly wider (P<0.001) when compared to *A. agama*. However, there were no statistically significant differences between the erythrocytes diameter (P>0.05) in *C. gariepinus* and *A. agama* (Figure 11). *C. gariepinus* had the least erythrocytes' volume (36.17±1.73 µm^3) compared to *A. agama* (61.50±2.66 µm^3) and *B. bufo* (112.40±7.22 µm^3). The erythrocytes' area in *B. bufo* was significantly higher when compared to *C. gariepinus* and *A. agama* (P<0.001). *C. gariepinus* erythrocytes were significantly lower (P<0.001) compared to *A. agama* (Figure 12). The volume of erythrocyte was 165.90±12.04 µm^3 in *C. gariepinus*, 920.60±88.49 µm^3 in B. bufo, and 453.60±24.51 µm^3 in *A. agama*. The volume of the erythrocyte of *A. agama* was significantly higher (P=0.001) when compared to *C. gariepinus.* The volume of erythrocyte of *B. bufo* was significantly higher (P<0.001) compared to *A. agama*. The volume of the erythrocyte of *C. gariepinus* erythrocyte volume was significantly lower (P<0.001) when compared to *B. bufo* (Figure 13).

4. Discussion

In the current study, the erythrocyte's shape ranges from spherical in *C. gariepinus*, oval to spherical in *B. bufo*, and oval in *A. agama.* Erythrocytes' shapes vary greatly in fishes, from oval in tilapia and *Channa punctata* [17, 18] to elliptical in (*Acipenser sinensis*)

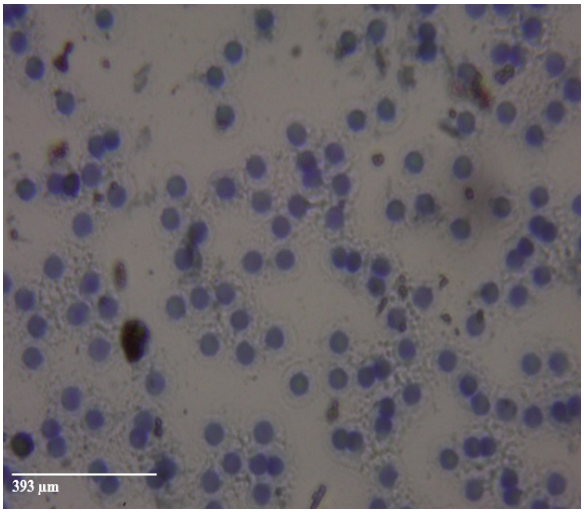

Figure 4. Erythrocyte of *C. gariepinus* stained with methylene blue x400 magnification, n=3

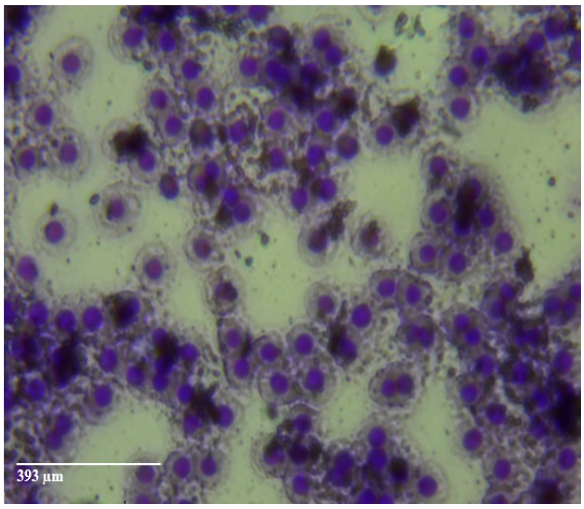

Figure 2. Erythrocyte of *C. gariepinus* stained with Giemsa, x400 magnification, n=3

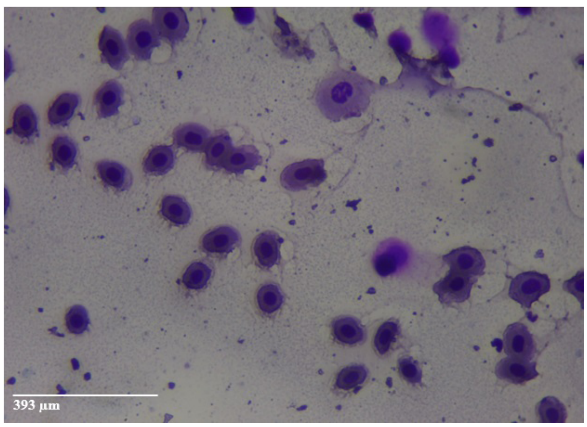

Figure 5. Erythrocyte of *B. bufo* stained with Giemsa, x400 magnification, n=3

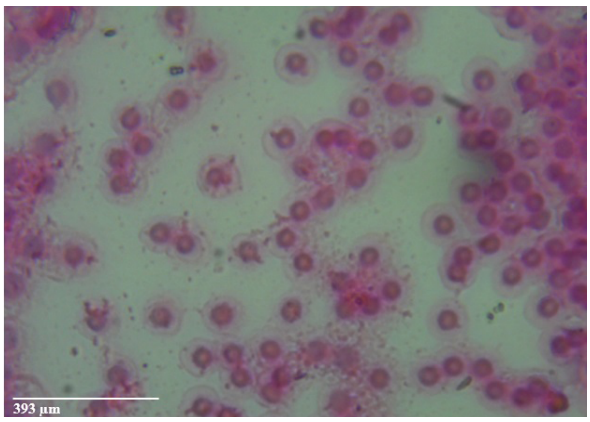

Figure 3. Erythrocyte of *C. gariepinus* stained with H&E, x400 magnification, n=3

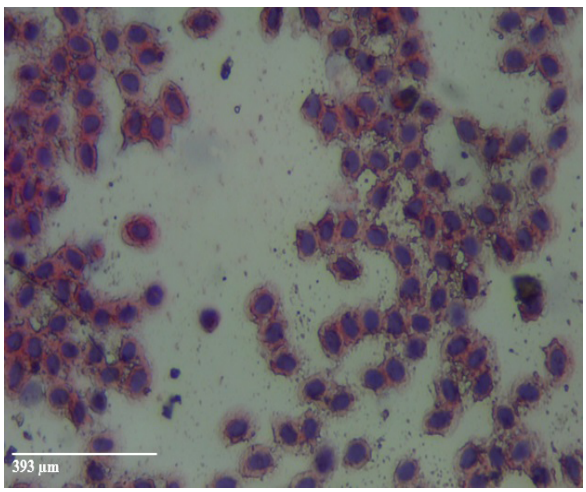

Figure 6. Erythrocytes of *B. bufo* stained with H&E, x400 magnification, n=3

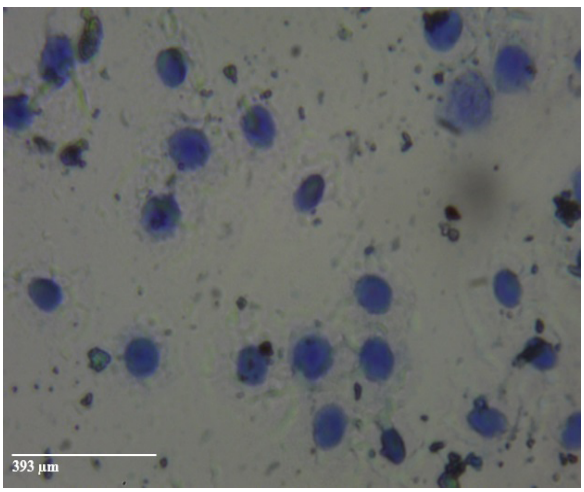

Figure 7. Erythrocytes of *B. bufo* stained with methylene blue, x400 magnification, n=3

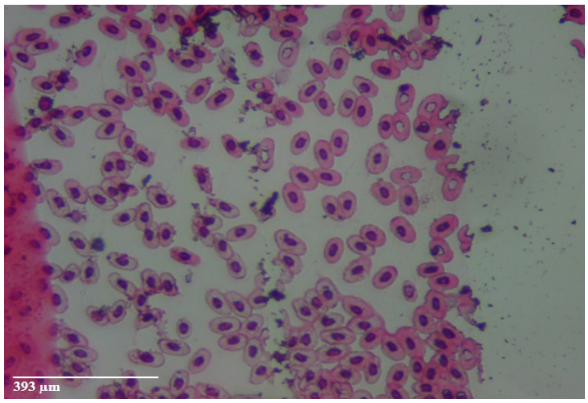

Figure 8. Erythrocytes of *A. agama* stained with H&E, x400 magnification, n=3

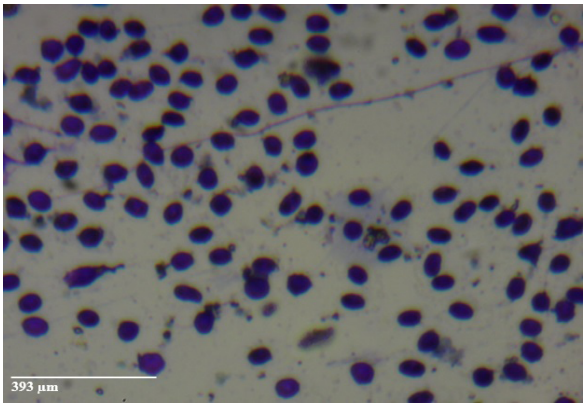

Figure 9. Erythrocytes of *A. agama* stained with Giemsa, x400 magnification, n=3

Chinese sturgeon [19]. In triploid species of *C. gariepinus*, erythrocytes are elliptical, while diploid *C. gariepinus* are round in shape [20]. In general, the erythrocytes of fishes vary from oval to elliptic, with few round shapes [21]. In amphibians, the erythrocyte's shape also ranges from oval in *Bufo gargarizans*, *Microphyla ornate*, *Rana esculenta*, and *Fejervarya limnocharis* [6, 22] to round in *Xenopus laevis* [23] and elliptical in *Bufo Vulgaris* [24].

Urodeles were reported to have more elongated erythrocytes than Anurans [25]. Circular and oval-shaped erythrocytes are found in the tadpole of Microphyla ornate and Indian tree frog [26, 27]. Erythrocytes of *A. agama* were similar in shape to other lizard species, Gollotia simonyi machadoi and Leiolepsis belliana rubritaeniata [28, 29]. The lizards of the genus Podarcis, Alggyroides, Zootoca, Timon, and other reptiles, including the red-eared slider turtle, all possess oval-shaped erythrocytes [30, 31].

The current study showed that the erythrocytes of *C. gariepinus*, *B. bufo,* and *A. agama* have distinct nuclei. Mature erythrocytes of non-mammalian species retain nuclei and some organelles responsible for energy supply and protein synthesis [32, 33]. The organelles in the erythrocytes of non-mammalian vertebrates include mitochondria, lysosomes, ribosomes, and endoplasmic reticulum [18, 34].

The erythrocytes' parameters (diameter, area, and volume) in *B. bufo* were significantly higher when compared with *C. gariepinus* and *A. agama.* The erythrocytes' size was reported to have a direct association with chromosome number in fishes [19]. Increased erythrocytes' size was noted with increasing chromosome number from diploid to triploid in *C. gariepinus* [20]. In vertebrates, erythrocytes' size is significantly correlated with genome size [35, 36]. Among vertebrates, amphibians tend to have the largest erythrocytes size [37]. This was also observed in the current study. Amphibians were reported to have the largest genome size and the most significant variation in genome magnitude ranging from 0.95 pg in *Platyplectrum ornatum* to 140 pg in *Necturus lewisi* [38].

The large genome size in amphibians might cause a higher diameter, area and volume of erythrocytes in *B. bufo* compared with *C. gariepinus* and *A. agama*. Reptiles generally have a small genome size ranging from 1.1 pg to 5.4 pg [35]. Hence, the smaller erythrocytes size in *A. agama* compared with *B. bufo*. In fishes, erythrocyte sizes might differ in different species due to genome size. The erythrocytes of Australian lungfish with a genome magnitude of 50 pg were about 10-fold higher when compared with Siamese fighting fish with a genome size of 0.6 pg [35]. Therefore, genome size is essential in determining erythrocytes' size in

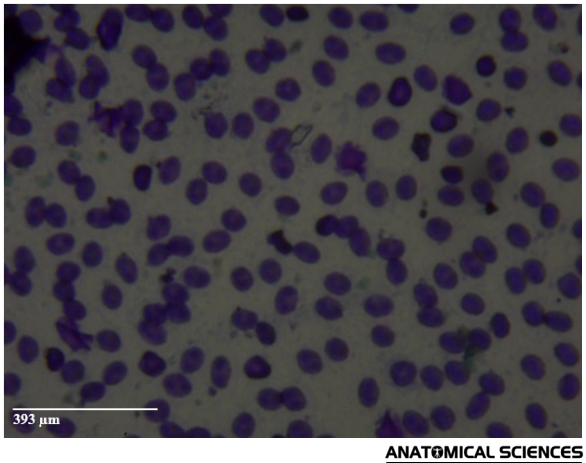

Figure 10. Erythrocytes of *A. agama* stained with methylene blue, x400 magnification n=3

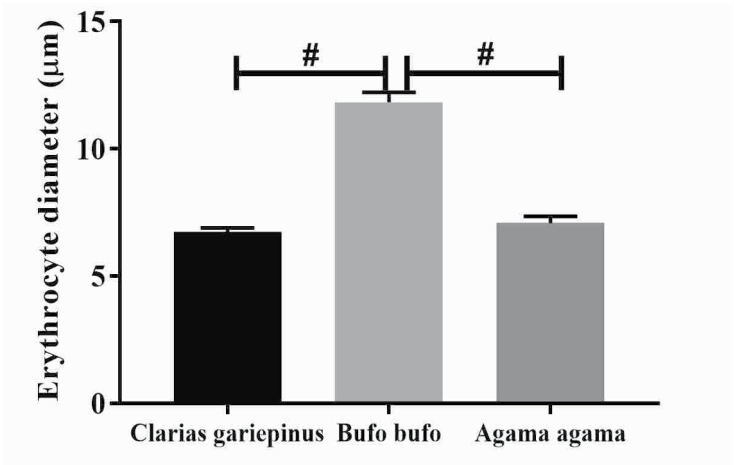

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Figure 11. Erythrocytes diameter in C. gariepinus, B. bufo, and A. agama
One-way ANOVA; #: indicates a significant difference at P<0.0001 n=3.

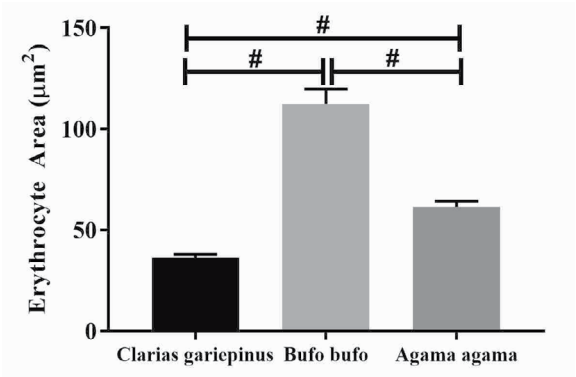

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Figure 12. Erythrocytes area in *C. gariepinus*, *B. bufo*, and *A. agama*
One-way ANOVA; # indicates a significant difference at P<0.0001 n=3.

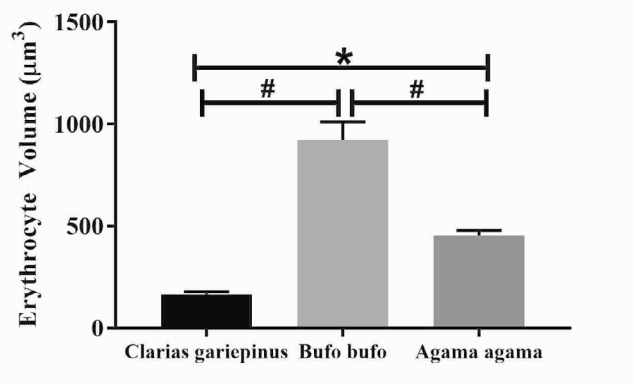

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Figure 13. Erythrocytes volume in C. gariepinus, B. bufo, and A. agama
One-way ANOVA; * indicates a significant difference at P=0.001 while # indicates a significant difference at P<0.0001 n=3.

different vertebrates and even the same vertebrates in a different genus.

5. Conclusion

The current study indicated that *C. gariepinus* and *B. bufo* erythrocytes were better demonstrated with Giemsa stain while *A. agama* erythrocytes were better shown with H&E stain. This signifies that Erythrocytes' affinity to stain varies in different vertebrates, probably due to environmental and genetic factors. The erythrocytes' shape, size, area, and volume varies in different vertebrates, and the variation is associated with chromosome number and genome size.

Ethical Considerations

Compliance with ethical guidelines

The research was approved by the Department of Human Anatomy Ethical Committee (Code: UM/HA/UGP19.20-095) and conducted in accordance with National research council guide for the use of laboratory animals (8th edition).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest to declare.

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