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Blood Cells Morphometry and Descriptive Morphology of Captive Changeable Hawk Eagles (*Nisaetus chirratus*) at Wildlife Rescue Centre Jogja

Andreas Bandang Hardian[1,2*], Warih Pulung Nugrahani[2], Irhamna Putri Rahmawati[2], Dorothea Vera Megarani[3]

[1]Laboratory of Veterinary Anatomic Pathology, Faculty of Veterinary Medicine Universitas Brawijaya
[2]Wildlife Rescue Centre Jogja, Yayasan Konservasi Alam Yogyakarta
[3]Department of Clinical Pathology, Faculty of Veterinary Medicine Universitas Gadjah Mada
*Correspondence: andreasbandangh@ub.ac.id
Jl. Puncak Dieng, Kunci, Kalisongo, Dau, Malang, East Java, Indonesia 65151
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ABSTRACT

Providing qualitative and quantitative haematologic references of neglected captive wild animals is pivotal for the sanctuary health management and improvement. Changeable hawk eagles (*Nisaetus chirratus*) are abundantly kept in sanctuary in which no haematologic reference is ever reported. This study aimed to present the visual keys and morphometric references of changeable hawk eagles' blood cells as the standard for further haematologic count. The peripheral blood smears were prepared and collected from eight changeable hawk eagles kept at Wildlife Rescue Centre Jogja then stained with 10-fold diluted Giemsa stain following the standard manners. All slides were inspected and captured under camera-equipped microscope which then were morphologically and morphometrically evaluated using ImageJ version 1.52a. As the changeable hawk eagles are naturally present in dark and bright morph, we statistically compared the blood cells morphometric parameters between morph-based groups. Changeable hawk eagles' erythrocytes were oval shaped with occasional morphologic variation. Leukocyte consisted of polymorphonucleated granulocytes - with exception of basophils which lacked nuclear lobulation - and mononucleated agranulocytes. There were significant differences ($P<0.05$) of all erythrocyte morphometric parameters, heterophils diameter, and lymphocytes diameter between dark and bright morph group. Overall, the morphologic properties of changeable hawk eagles' blood cells were visually identical to other avian species though the blood cells morphometry might be comparatively different.

Keywords: erythrocyte, haematology, leukocyte, measurement, raptors

ABSTRAK

Ketersediaan referensi hematologi berupa data kualitatif dan kuantitatif dari spesies satwa liar terabaikan di fasilitas rehabilitasi adalah hal penting untuk manajemen dan peningkatan mutu kesehatan hewan di dalam suaka. Elang brontok (*Nisaetus chirratus*) telah banyak dilaporkan berada di fasilitas eksitu tanpa adanya referensi hematologi untuk spesies ini. Studi ini bertujuan untuk mengenalkan panduan visual dan referensi morfometri sel-sel darah elang brontok sebagai standar untuk pemeriksaan hematologi kuantitatif selanjutnya. Preparat apus darah dibuat dari delapan ekor elang brontok dari *Wildlife Rescue Centre Jogja* yang kemudian diwarnai dengan pewarna Giemsa. Seluruh preparat diinspeksi dan difoto menggunakan kamera mikroskop untuk selanjutnya dievaluasi secara morfologis dan morfometrik menggunakan ImageJ versi 1.52a. Kami membandingkan parameter morfometrik sel darah dari dua kelompok elang brontok berdasar dua fasenya: fase gelap dan fase terang. Eritrosit elang brontok berbentuk oval dengan beberapa variasi morfologi. Leukosit elang brontok terdiri atas granulosit polimorfonuklear - kecuali basofil yang tidak memiliki lobulasi nukleus - dan agranulosit mononuklear. Terdapat perbedaan signifikan ($P<0,05$) pada semua parameter morfometrik eritrosit, diameter heterofil, dan diameter limfosit antara kelompok elang fase terang dan fase gelap. Gambaran morfologi sel darah elang brontok secara visual identik dengan spesies burung lain walaupun morfometri sel darah memiliki perbedaan.

Keywords: eritrosit, hematologi, leukosit, pengukuran, raptor

INTRODUCTION

Changeable hawk eagles (*Nisaetus chirratus*) are the most common terrestrial raptor species found in captivities in Java, Indonesia. Their habitat ranges from lowland to mountainous areas across Java, although they may compete with other raptors for territory and preys (MacKinnon *et al., 2010*) . These eagles are present into two morphs of color: dark dan bright morphs. While others occasionally categorize them into three morphs–including intermediate morphs, which makes the former bright morphs are more identical to their relatives, Flores eagle (*Nisaetus floris*)-, the cause of this polymorphic phenomenon is still poorly understood. However, the latest taxonomy classified them as the same species, suggesting the presence of encoding gene polymorphisms of feather phenotypes and possibly a subspeciation event.

The high number of individuals sent to the rescue centers and zoos due to law enforcement against illegal trade and ownership indicates a frequent raptor trafficking of this species. Approximately 136 individuals of changeable hawk eagles were kept in nine zoos and rehabilitation centres in Java in 2019. Greater attention and conservation effort have been dedicated to their relative – Javan eagle (*Nisaetus bartelsi*)–which may explain the reason why the chance of getting a prospective release site for Changeable hawk-eagle species is quite low in Java. While the rehabilitation takes place in a captive facility such as a rehabilitation center, preventive health management such as routine health examination complemented with several laboratory tests needs to be carried out to maintain the health of the birds. However, data on clinical parameters of these neglected raptors are very deficient. Therefore, providing a clinical parameter reference is necessary.

There is scarce information about the reference of health parameters of wild animals. Medical information is mostly present as individual scattered data which might be standardized. Furthermore, most of the time, the veterinarian is challenged with the limited equipment available to carry out a laboratory test. Therefore, only a clinical examination with basic laboratory tests can be performed, such as a fecal and hematologic test, depending on the available equipment in the field. The conventional hematologic test, like a complete blood count, is easy to perform and can reveal numerous indicators of general health conditions (Monks & Forbes, 2007; Jones & Chitty, 2020). Nevertheless, a descriptive morphology of each white blood cell line for differential leukocyte count in one species needs to be determined (Campbell, 2015; Jones & Chitty, 2020). This is especially so in our case, when the small lymphocyte size in eagles is identical to their solitary platelet size, which may obscure the cell determination during the total white blood cell counting.

A changeable hawk-eagle is one of many raptor species in which none of the normal physiological and clinical references is ever reported. The closely related hematological studies of this genera are only a case report of blood parasitism in a Blyths-hawk eagle (*Nisaetus alboniger*) and a study of hematological and biochemical properties of Javan eagle (*Nisaetus bartelsi*) (Pornpanom *et al*., 2019; Santosa *et al*., 2003). Several metabolic alterations including poisoning and toxicosis evidently affect morphology of blood cells in birds (Mitchell & Johns, 2008). Chronic inflammation, anemia, blood parasite infestation were also reported altering the hematology of birds (Mitchell & Johns, 2008).This study aimed to explore the morphology of changeable hawk eagle's blood cells in quantitative and descriptive aspects. The result of this study provides a reference to count total red and white blood cells as well as a differential leukocyte.

MATERIAL AND METHOD

Permission and clearance

This study was conducted under the legal permission of the Indonesia Ministry of Environment and Forestry (certificate No. SK.353/KSDAE/SET/KSA.2/8/2019). All methods of this study have been approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Gadjah Mada (certificate No. 0124/EC-FKH/Eks./2019).

Blood smear preparation

Blood sample collection was done in conjunction with the annual health examination at the center. Eight changeable hawk individuals consisting of four individuals of dark morph and four individual of bright morph were used in the study, all presented with negative to blood parasites and endoparasites. Two-mililiter blood was collected from the brachial vein of each bird using a standard protocol (Monks & Forbes, 2007; Hawkins *et al*., 2001).Two blood smears for each bird were prepared to evaluate the morphology of blood cells. Methanol was used as a fixative agent. Giemsa stock (*Giemsa's azur eosin methylene blue solution*, Merck KGaA, Darmstadt, Germany) was diluted with distilled water using a ratio of 1:9 for staining. The fixated blood smears were then soaked in diluted Giemsa stain for 10-15 minutes and were rinsed subsequently with tap water. At last, Giemsa-stained blood smears were drained to dry at room temperature.

Cell morphometry and morphologic description

The morphology observation and cellular morphometry were carried out under a camera-equipped microscope (Optilab Professional camera, PT Miconos, Yogyakarta, Indonesia) with 100-time magnification. The cytological screening was done with the battlement technique. Observation of at least 15–30 cells of each blood cell type was started from the area with moderate erythrocyte density (approximately 20–50 cells per field). The determination of cell morphology referred to the eagle blood cell description suggested by Salakij *et al.*, (2015a).

Cell morphometry was done by initially taking a photo of the cells that were set aligned with the calibration slide, using a microscope camera generating a resolution of 2560 x 2048 pixels. ImageJ version 1.52a was calibrated into 24.8 pixels per µm using a DIV 0.01 calibration slide (Optilab Advance, PT Miconos, Yogyakarta, Indonesia) before measuring the cellular morphometry parameters. Erythrocyte morphometry parameters were cell length, cell width, nucleus length, and nucleus width, while the spherical leukocyte was only the cell diameter or longest axis.

Statistical analysis

All statistical descriptions and comparisons of cellular morphometry were analyzed using R version 3.3.3 (R Core Team, 2017). The Shapiro-Wilk and Lilliefors normality test and Levene's homogeneity test were performed to check whether the data met parametric criteria. The comparison between the two parametric groups was tested using two-sample t-test (α=0.05). Wilcoxon rank-sum test (α=0.05) was applied if the data were not parametric.

RESULT

Digital cell morphometry using ImageJ was done in 240 erythrocytes, more than 100 leukocytes excluding basophil, and more than 200 thrombocytes. Different staining methods of several avian blood cells applied in various literature might visually obscure cell identification. Hence, the cell determination in this study was based on the common structure and component of avian blood cells, such as nucleus shape and cytoplasmic granules. The morphometry results were presented in Table 1, 2, and 3. The analysis of leukocytes' longest axis revealed that the monocyte was the largest cell, while basophil appeared to be the smallest one (Table 2). There was a significant difference in morphometry parameters between the two groups.

Erythrocytes were oval with clear cytoplasm (Figure 1a). The nuclei were stained purple to magenta with distinct dark-blue chromatin. The circulating RBC-like cells were found occasionally, which were later identified as the immature erythrocytes. These immature erythrocytes visually possessed a bluish cytoplasm with a relatively larger nucleus compared to the mature ones.

The heterophils showed no distinct cytoplasmic granule. Shapes were commonly spherical, although amoeboid figures might occasionally appear. The nucleus was stained dark purple due to the dense chromatin containment, visually segmented with several narrowings, and two to four lobulations might be seen. The cytoplasm was stained pale pink with the faded fine granules scattered around it. Although the cell size was identical to eosinophil, both cells were easily distinguished based on the apparent morphology.

Eosinophils might be the most visually prominent leukocyte due to the presence of densely packed reddish granules in their cytoplasm (Figure 2b). The cells were mostly spherical. The nucleus mainly appeared bi-lobulated with a very narrow belt, which made it looked like purple twin teardrops. The compact reddish granules stuffed the cytoplasm, hence sometimes protruded from the cell membrane. Although a variety of eosinophil sizes was found, the reddish granules were still distinctive to be a marker.

Basophils appeared bright-magenta-stained small irregular to spherical shape with the indistinct round nucleus and occasional dark purple cytoplasmic granules protruding to the cell membrane (Figure 2c). Almost none of the basophil's cytoplasmic compartment was seen under Giemsa staining. The whole nucleus was rarely seen due to the dense coverage of purple cytoplasmic granules. Basophils were the rarest leukocyte found in this study. The shape ranged from irregular, ovoid to spherical. Morphometry revealed that these cells were the smallest cell among other leukocytes.

A lymphocyte contained a magenta-stained round nucleus with the coarse dark-purple chromatins (Figure 3a). The cytoplasm was stained clear grey and less basophilic than the monocyte cytoplasm. The cytoplasmic compartment was tighter compared to the nucleus. Some lymphocytes were occasionally found bursting. However, most cells remained relatively intact and distinctive. No cytoplasmic granule was seen.

Monocytes appeared as large cells with dark purple ovoid to round bi-lobulated nuclei (Figure 3b). Dark blue spots were occasionally encountered, indicating the clumps of chromatins. The narrowing between

lobules was bigger than in the polymorphonuclear cells, which resembled a nucleus fold rather than segmentation. The cytoplasmic compartment was prominently larger than the nucleus and pale basophilic. The shape was spherical to amoeboid with no distinct granule was present.

Thrombocytes or platelets were mostly found in the aggregate consisting of several cells, although solitary platelets were occasionally seen (Figure 1c). The shapes were ovoid with magenta to the purple nucleus. Platelet morphometry was done primarily for nucleus length because there was no apparent cell membrane of platelet clumps found in most blood smears (Figure 3c). However, several clusters of platelet with the intact cell membrane were still found (Figure 4).

The variation of erythrocyte morphology and size in the changeable hawk eagles includes the presence of immature erythrocytes, macrocytes, binucleated erythrocytes, and uncommon erythroplastids. Immature erythrocytes were present in almost all screened slides and were distinguished by the more rounded cells and nucleus with less basophilic cytoplasm (Figure 5-w). Two erythroplastids were found in two eagle individuals (Figure 5-v). These cells were the anucleated erythrocytes, appeared as an empty oval-shaped avian erythrocyte.

DISCUSSION

Several studies reported that the morphometry of avian erythrocyte might differ among birds due to the variation of activity, daily diet, genetic, and environmental conditions. The higher level of exercise demands more circulating oxygen. Otherwise the bird may suffer from hypoxia. While the endemic raptors such as Javan eagles are likely to soar within their territory (Kaneda *et al*., 2009; Nijman & van Balen, 2003), migratory birds have evolutionarily adapted their oxygen supply mechanism during the long intercontinental migration by expanding the oxygen carrying capacity and improving the properties of blood cells (Elarabany, 2018; Adediran *et al*., 2015). Some raptor species may undergo local migration through the shorter flyway; however, they do not suffer from a drastic environmental transition like the longer-flyway-migratory birds do. Habitat altitude and terrain type (coastal or terrestrial) may affect the blood cell properties due to the altitude-associated-oxygen level or prey availability (Adediran *et al.*, 2015; Scott, 2011; Yap *et al.*, 2018). The type of diet may associate with the hemoglobin replenishment in response to the need of iron-containing-food and the supports toward hematopoietic organs to regenerate blood cells in certain conditions like hemolytic anemia (Minias, 2015; Capitelli & Crosta, 2013).

All immature erythrocytes found were present as the latest developing polychromatophilic erythrocytes. These morphologies were consistent with the description in the previous studies (Campbell, 2015; Clark & Raidal, 2014). Nonetheless, Captelli & Crosta reported that immature erythrocytes might appear in various stages (Capitelli & Crosta, 2013). The novice hematologists may find difficulties distinguishing the avian large mononuclear agranulocytes, particularly on whether it is a rubriblast or monocyte. However, rubriblasts and their derivatives must not be mistaken as monocytes because the rubriblast's cytoplasm is stained more basophilic than the monocytes, which were stained more transparent.

Anisocytosis is considered common at a low number (less than five) in the peripheral avian blood (Campbell, 2015; Capitelli & Crosta, 2013). An increased number of anisocytosis is considered clinically pathologic, which reflects the conditions such as regenerative anemia, blood parasitism, and toxicosis or vascular disturbance (Prokić *et al.*, 2019; Latimer, 2011). The presence of erythroplastid is considered common (Campbell, 2015; Capitelli & Crosta, 2013) and has been reported in several raptor species such as a little falcon (*Falco longipennis*), a peregrine falcon (*Falco peregrinus*), and an Australian masked owl (*Tyto novaehollandiae*) (Clark & Raidal, 2014). To date, there is no study reporting about the meaningful clinical significance of erythroplastid occurrence in a peripheral blood slide. However, Clark and Raidal (2014) found that the concentration of erythroplastid was poorly correlated to the packed cell volume (PCV) of 30 birds, thus further researches about erythroplastid formation and its significance on clinical hematology is needed.

The changeable-hawk eagles' erythrocytes and leukocytes morphometry revealed the cell size difference compared to six other southeast-Asian eagles: crested serpent eagle (*Spilornis cheela*), shikra sparrow hawk (*Accipiter badius*), Blyth-hawk eagle (*Nisaetus alboniger*), black shouldered kite (*Elanus caeruleus*), Brahminy kite (*Haliastur indus*), and black kite (*Milvus migrans govinda*) (Salakij *et al*., 2015a, 2015b, 2019). The erythrocyte morphometric parameters of the dark morph changeable-hawk eagles were closely similar to the Blyth-hawk eagle and crested serpent eagle. The varying leukocyte size among raptors species might reflect the cell reactivity or inflammatory responses at the time of collection.

The statistical comparison of erythrocyte and leukocyte morphometry between the dark and bright morphs of changeable hawk eagle showed significant

differences. All erythrocyte parameters between the two groups showed a significant difference. The heterophil, basophil, and lymphocyte also showed a significant difference, associating with the cells' morphological character. Heterophils were generally found in amoeboid shape, while the eosinophils in both groups tend to be spherical. Avian lymphocytes were generally present in various sizes. The diversity of the shape of the cell resulted in a high variation of cell diameter. The significant differences of erythrocyte and lymphocyte morphometry among breeds were previously reported in the white and bronze turkeys (Bhattacherjee *et al*., 2017). In spite of the unclear physiological consequences, the morphometric differences among avian breeds in the same species may reflect the breed-specific hematological features.

Heterophils and eosinophils seemed to be the only polymorphonucleated granulocytes screened from the eagles' peripheral blood slides. The staining method using only Giemsa solution seemed to affect the coloration of heterophils' cytoplasmic granules. In several studies of raptor hematology, the combination of Giemsa and Wright stain visually distinguished the heterogenicity of the granules' shape and color (Salakij *et al*., 2015a, 2019). Meanwhile, the diluted Giemsa-only solution resulted in a weak and faded coloration to the granules of raptor heterophils, creating a clear pinkish cytoplasm seen in the mammalian neutrophils. A similar staining solution also yielded a distinct vibrant red granule presentation of eosinophil as reported in the previous studies (Salakij *et al*., 2015a). Similar to other references, the basophil of the eagle in this study were mononuclear cells with dark purple granules packed densely within the small space of cytoplasm (Campbell, 2015; Salakij *et al*., 2015a). However, the low number of basophil in this study was apparently contradicted the fact that birds usually possess a considerable amount of basophil population in peripheral blood circulation (Campbell, 2015). That condition could be caused by an error cell recognition due to the indistinct visual characteristic of avian basophil, or that is a native trend in changeable hawk-eagle hematology. Furthermore, Salakij et al. reported that even the same leukocyte lineage among bird species might have a diverse enzymatic activity, indicating that each bird species might have its own hematological trend (Salakij *et al.,* 2015a).

Birds were reported possessing three types of lymphocyte according to the cell size: small, medium, large (Campbell, 2015; Capitelli & Crosta, 2013). Here we roughly categorized the changeable-hawk eagle lymphocyte size based on the visual inspection of the histogram and strip chart into three groups (Figure 6): small (< 6 µm), medium (6 - 8.5 µm), large (> 8.5 µm). The lymphocyte size variation was also present in reptiles (Jacobson, 2017). However, it is still unclear whether the size variation of lymphocyte indicates their reactivity, or it is certainly the three different functional cell types that emerged from a single lymphoid progenitor lineage. The antigenically sensitized lymphocytes are relatively larger than the naive lymphocytes because of the numerous antibody containment produced inside their cytoplasm (Campbell, 2015; Capitelli & Crosta, 2013; Latimer, 2011). The diversity of mammalian lymphocyte size comparatively gave a clearer presentation about its functional properties by being classified into a natural killer cell, T-, and B-lymphocyte (Weisser, 2012). Lymphocyte was broadly described as the most abundant leukocyte in birds and some animals in lower taxa (Davis *et al*., 2008). Thus, the presence of size variation and the considerable number of lymphocyte in peripheral avian blood circulation may account for some certain functional properties.

The right method to either morphologically or morphometrically distinguish the avian platelets, basophils, and small lymphocytes, especially under the counting chamber, is still problematic. In our experience, the morphometric and morphologic features of those cells did not elicit any significant difference after being stained with the common Natt-Herrick or Dixon's modified Rees-Ecker solution. The rules of thumb to recognize those cells, such as by appreciating the clumping cells, which specifically referred to platelet, might still be applicable. The Giemsa staining had allowed us to perform morphological recognition of blood cells,. However, several solitary platelets found in the peripheral blood smear could be mistaken as small lymphocytes under the counting chamber.

The common Giemsa staining method may not be the best, but it was sufficient to differentiate each leukocyte lineage in this study. Several avian hematology studies suggested the better staining method for peripheral blood smear examination, such as the combination of Wright and Giemsa solution and Diff-Quick solution (Campbell, 2015; Salakij *et al*., 2015a, 2019). Those staining methods produced a higher color contrast among the background, cytoplasm, and nucleus of erythrocytes, thus creating a distinct pinkish cytoplasm of the cells (Campbell, 2015). Nevertheless, it might be laborious to achieve the correct mixture composition from both solutions. A critical time of incubation should also be managed properly because the longer time the blood smear immersed in the dye solution, the darker the stained cells would be. Using the Wright-Giemsa staining solution is an excellent method for examining the peripheral blood smear,

but not all raptor rehabilitation facilities or sanctuaries have many choices on the staining solution. It has also been advised that the blood fixation using methanol might occasionally induce lymphocyte bursts (Chaleow Salakij – personal communication).

Overall, the blood cell morphological properties of the changeable hawk-eagle are visually identical to other avian species but morphometrically diverse. The clinical hematology provides a sufficient initial information about the avian health status, in line with

Table 1. The morphometry of changeable-hawk eagles' erythrocytes

Erythrocyte	Dark morph (µm)	Bright morph (µm)
Cell length	12.88 ± 0.55	12.67 ± 0.74*
Cell width	7.18 ± 0.53	6.72 ± 0.54*
Nucleus length	5.66 ± 0.53	5.35 ± 0.57*
Nucleus width	2.51 ± 0.3	2.62 ± 0.22*

*Significant difference between groups at P<0.05.

Table 2. The morphometry of changeable-hawk eagles' leukocytes

Leukocyte	Dark morph		Bright morph	
	N	Mean±SD (µm)	N	Mean±SD (µm)
Heterophil	118	10.02 ± 0.86	120	10.53 ± 0.77*
Eosinophil	106	10.13 ± 0.91	118	10.47 ± 0.91*
Lymphocyte	99	7.12 ± 0.95	109	7.56 ± 0.94*
Monocyte	92	11.01 ± 1.73	106	10.99 ± 1.23
Basophil	5	6.69 ± 0.66	0	0

*Significant difference between groups at P<0.05.

Table 3. The morphometry of changeable-hawk eagles' thrombocytes

Thrombocyte	N	Mean±SD* (µm)	Min (µm)	Max (µm)
Cell length	31	7.33±1.28	5.56	9.88
Cell width	31	4.78±0.39	4.2	6.06
Nucleus length	233	4.47±0.48	3.31	5.85
Nucleus width	31	3.7±0.33	3.07	4.67

*standard deviation (SD)

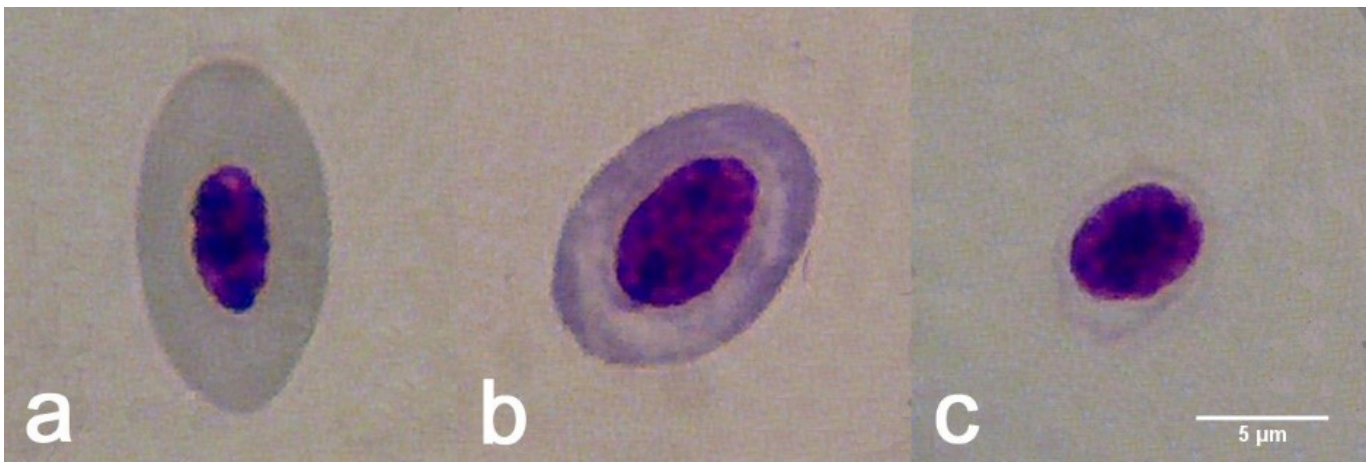

Figure 1. The visual appearance of mature erythrocyte (a), immature erythrocyte (b), and solitary platelet (c) showed differences in shape and color.

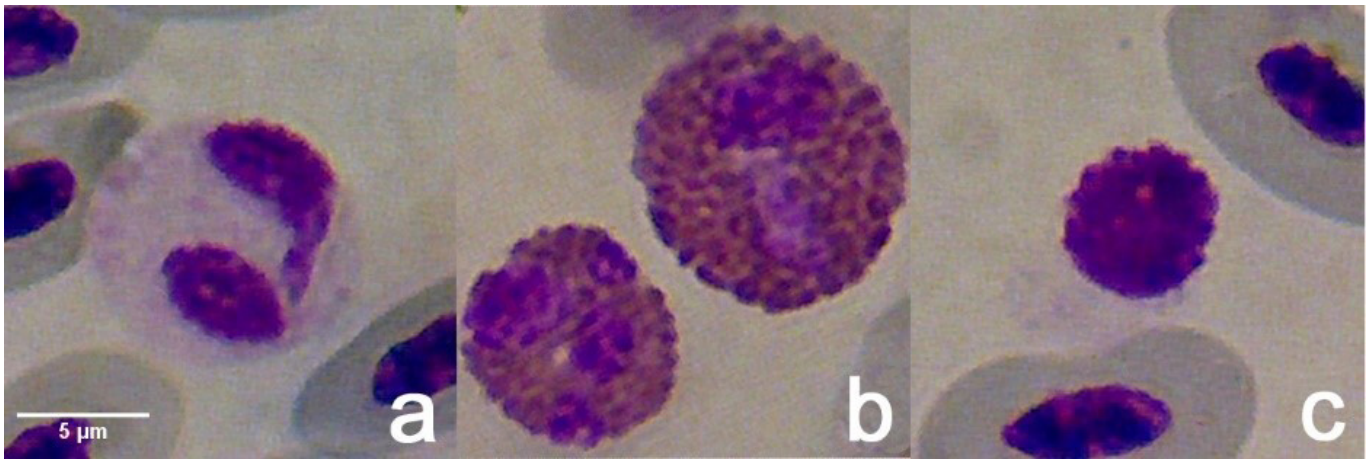

Figure 2. The comparative morphology of mature circulating heterophil (a), eosinophil (b), and basophil (c) showed the various cytoplasmic granules distinction.

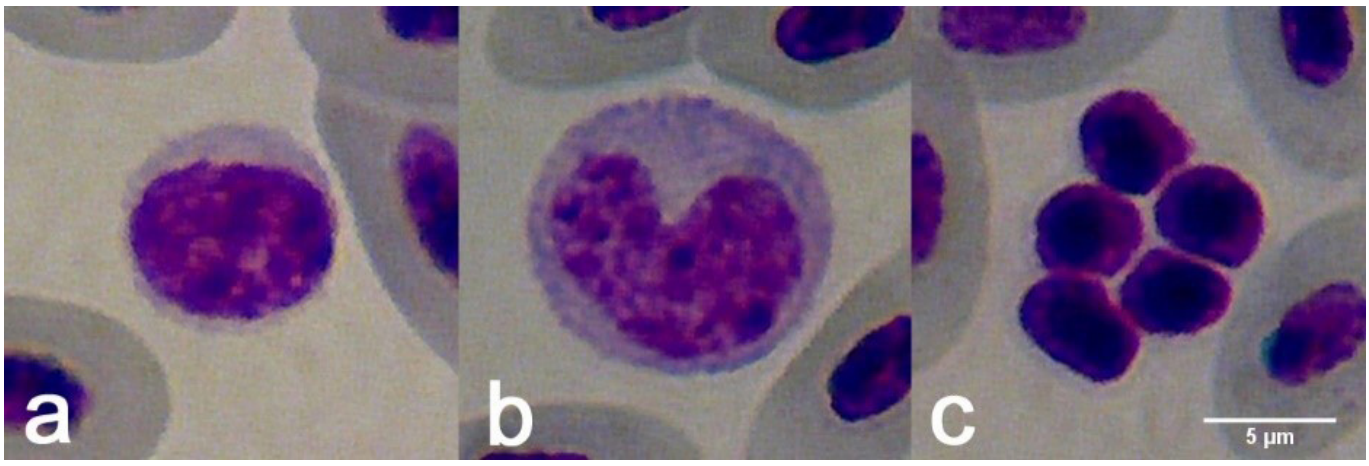

Figure 3. The light micrograph of a circulating lymphocyte (a), monocyte (b), and platelet clump (c) depict a difference in cell size.

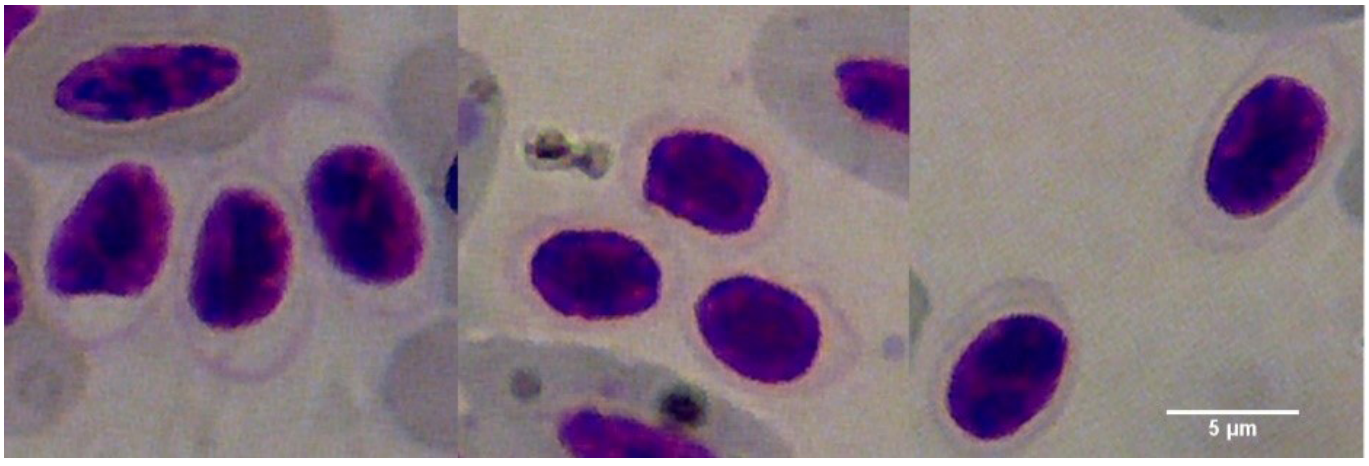

Figure 4. The circulating platelets were aggregated in a clump containing several cells.

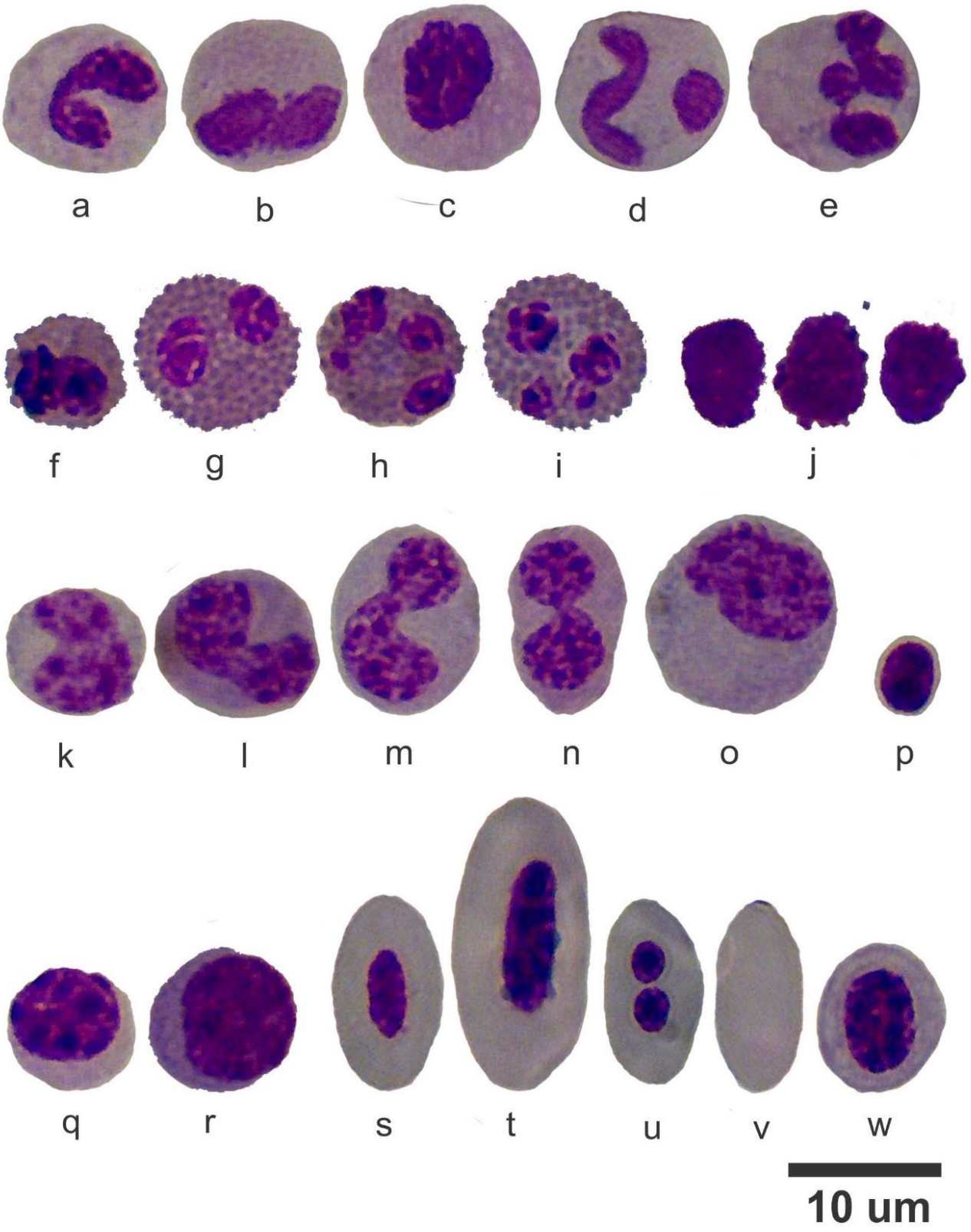

Figure 5. The changeable-hawk eagle blood cells' morphologic variation in their comparable actual size and Giemsa stained color: heterophils (a-e), eosinophils (f-i), basophils (j), monocytes (k-o), thrombocyte (p), lymphocytes (q,r), mature erythrocytes (s), large erythrocyte (t), binucleated erythrocyte (u), erythroplastid (v), immature erythrocyte (w).

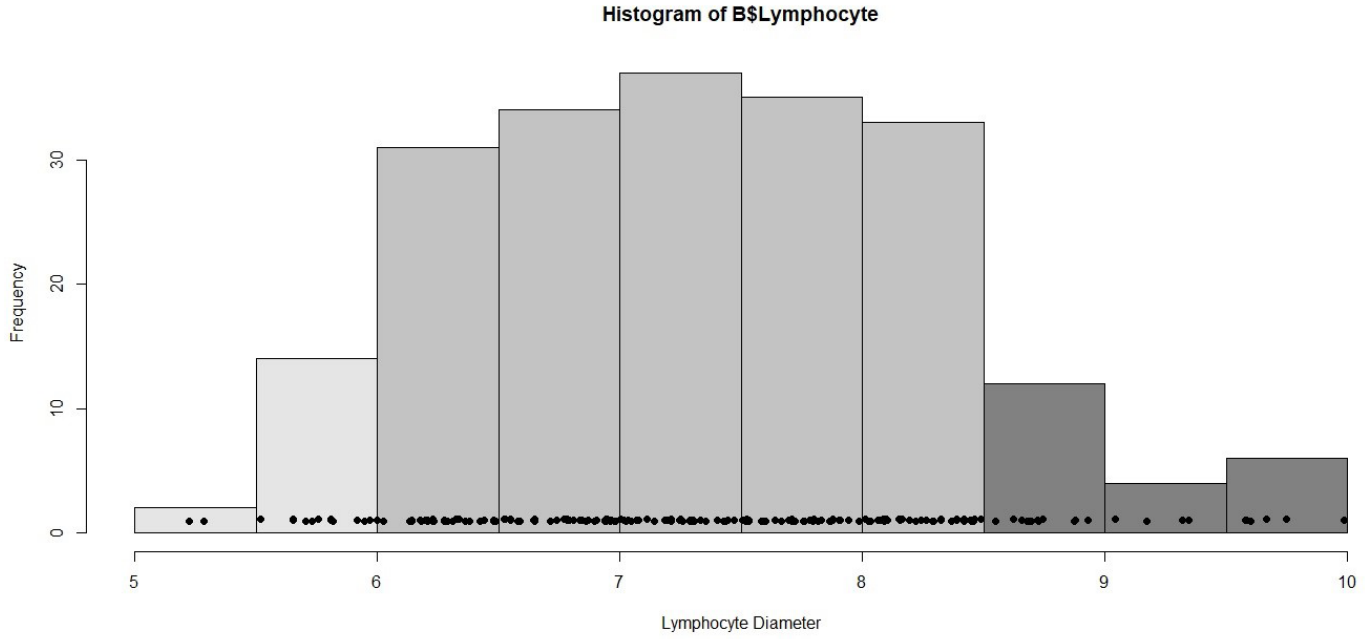

Figure 6. The histogram and strip chart comparing the occurrence frequency and diameter of the changeable hawk eagle's lymphocyte.

the physical examination. Given the fact that there is a diverse characteristic of blood cells among birds, understanding the morphology and morphometry of certain avian blood cells might be helpful to be able to identify each cell line when performing hematology examination. The dynamics of erythropoiesis and myelopoiesis might contribute to the ultrastructural properties and enzymatic activities of avian leukocytes and erythrocytes. Furthermore, Stier *et al.* (2013) suggested that the evident functional mitochondria in avian erythrocytes reveal a hint to other fascinating properties of avian blood cells.

The changeable hawk eagles' blood cells were morphologically similar to other avian species, while the blood cells morphometry apparently heterogeneous among raptors. The cytochemical and ultrastructure studies may reveal more about the blood cell properties of these eagles. At last, establishing a hematology reference interval of these eagles with sufficient sample size is necessary as the clinical determination standard of the eagle's health status.

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"The authors state that they have no known conflicting financial interests or personal ties that may be seen as having influenced the work described in this study."

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