Research Article

Blood Cell Profile of the Developing Tadpoles and Adults of the Ornate Frog, *Microhyla ornata* (Anura: Microhylidae)

Jutshina Hota, Madhusmita Das, and Pravati Kumari Mahapatra

P. G. Department of Zoology, Cell and Developmental Biology Laboratory, Utkal University, Bhubaneswar, Odisha 751 004, India

Correspondence should be addressed to Pravati Kumari Mahapatra; mahap_pk@yahoo.com

Received 31 May 2013; Revised 30 July 2013; Accepted 5 August 2013

Academic Editor: Roger P. Croll

Copyright © 2013 Jutshina Hota et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Metamorphosis happens to be an important event in the lifetime of amphibians. Our study offers a record of blood cell profile of laboratory reared tadpoles during development and metamorphosis (Gosner stage 26 to 46) and adults of *Microhyla ornata*. The larval erythrocytes were observed to be circular, oval, and elliptical in shape. However, other variations were distinct during the prometamorphic and metamorphic stages. Crenulated erythrocytes showed a pattern of appearance, and the crenulations varied from minute serrations to highly spiked projections. Correlations between the morphometric values of erythrocytes during the larval development were also determined. The leukocyte profile of the tadpoles showed a high percentage of lymphocytes during larval development while the percentage of monocytes, eosinophils, and neutrophils remained high during metamorphosis and were positively correlated with the developing stages. Blood thrombocytes of the tadpoles were small and were found in clusters. Elliptical erythrocytes were the most common in the adult frogs. However, few erythrocytes were also circular in shape. In adults, the percentage of lymphocytes was found to be more in comparison with the other leucocytes, and neutrophils showed various polymorphic forms. Thrombocytes were nucleated and spindle shaped.

1. Introduction

Metamorphosis in amphibians is often a time of dramatic developmental change affecting nearly the entire organism and has been a subject of investigation in several directions [1, 2]. During this time, a large proportion of the animal's structure changes, the larva, and adults are unrecognizable as being the same individual. Interestingly, these two phases have opposing effects on tissues as in the first phase, there is growth and development of tissues and increase in body size, and in the second phase, massive tissue reconstruction and breakdown leading to a reduction in overall body size occurs [3]. This brings about changes morphologically, physiologically and behaviorally to prepare for a new mode of existence.

Herpetologists are becoming increasingly aware of the importance of hematological parameters for evaluating the welfare of their study animals [4]. Interpretation of the hematological parameters offers valuable pieces of information concerning the health status of the organism [4]. The erythrocyte size has also been described to be used in

ploidy determination [5]. The effects of the phenomenon of tissue growth and lysis on the relative distribution of white blood cells in circulation have been considered as an important biological phenomenon since the 1920s [3], and a growing number of ecologists are turning to the enumeration of white blood cells from blood smears to assess stress in animals [4]. Recent interest in counts of leucocytes in amphibians for environmental monitoring emphasizes the need to understand how white blood cells naturally vary throughout larval life [6–9]. There also exists considerable variation in cell morphology in amphibians due to variation in metabolism [10–12].

Since normal hematology of many species is poorly understood and reference values are scarce [13], the present study aims to investigate the blood cell profile of the laboratory reared tadpoles and adult frogs of *Microhyla ornata* (Duméril and Bibron, 1841) inhabiting, Nandankanan, Bhubaneswar (20°24'10"N, 85°48'13"E), Odisha, India. *Microhyla ornata* is a microhylid frog belonging to class anura. A considerable work on *M. ornata* has been done with respect to life history [14], red blood cell sizes of adults

FIGURE 1: Adult frog and developmental stages of the ornate frog, *Microhyla ornata*. (a) Adult *M. ornata*, (b) premetamorphic tadpole (Gosner stage 28), (c) premetamorphic tadpole (Gosner stage 31), (d) prometamorphic tadpole (Gosner stage, 36), (e) prometamorphic tadpole (Gosner stage 39), (f) prometamorphic tadpole (Gosner stage 40), (g) metamorphic stage (Gosner stage 42), (h) metamorphic stage (Gosner stage 43), (i) metamorphic stage (Gosner stage 45), and (j) metamorphic stage (Gosner stage 46). Scale bar = 5 mm.

[12], toxicities of some heavy metals [15], morphological, and acoustic comparisons between *M. ornata*, *M. fissipes*, and *M. okinavensis* [16], taxonomic relationships using mtDNA analysis [17], and changes in polyamine contents [18]. However, hematological study of both tadpoles and adult frogs remains unexplored in this species in India. Study of hematological parameters during premetamorphic, prometamorphic, and metamorphic stages is crucial to gain proper understanding of changes occurring in the blood cells during metamorphosis; as change in mode of life brings about several new challenges. This paper provides the record of blood cell profile during development and metamorphosis in the tadpoles and also adult frogs of *M. ornata*.

2. Materials and Methods

2.1. Collection of Egg Nests and Adult Frogs. Egg nests and adult frogs of *Microhyla ornata* were collected from their natural habitat. The egg nests were kept in plastic tubs containing conditioned 20 mm deep tap water (tap water stored and aerated for 72 hours). The tadpoles were reared following standardized procedure [19] and were fed with yolk of boiled egg of *Gallus gallus domesticus* (breed White Leghorn) *ad libitum*. The adult frogs (Figure 1(a)) after collection were maintained in the terrarium for acclimatization to laboratory conditions. All procedures were approved by the Animal Care Review Committee at Utkal University.

2.2. Tadpoles Investigated. For the study, tadpoles from Gosner stages 26 to 46 [20] were considered which were comparable to the Taylor and Kollros [21] stages I to XXV. Ten numbers of tadpoles for each stage were selected for investigation. The tadpoles were divided into three subgroups based on developmental periods that are, premetamorphic (stages 25 to 35) (Figures 1(b) and 1(c)), prometamorphic (stages 36 to 41) (Figures 1(d), 1(e), and 1(f)), and metamorphic (stages 42 to 46) (Figures 1(g), 1(h), 1(i), and 1(j)) according to McDiarmid and Altig [22]. Prior to drawing blood for peripheral smear, snout to tail tip length (STL) of tadpoles and snout to vent length of metamorphosed froglets were measured.

2.3. Preparation of Blood Smears. Procedures described by Das and Mahapatra [23] were followed. Blood samples for adult frogs were successfully collected following standardized methods [24] without harming the animals in the morning hours. Tadpoles were anesthetized by exposing them to 0.3% MS-222 (Tricaine Methane Sulphonate) solution. The blood of tadpoles from stage 26 to 44 was obtained from tail amputation through the middle of the tail. For stages 45 and 46, blood was collected from the heart using a 26 gauge syringe needle. Blood smears were prepared using push slide technique. The dried blood smears were stained with Giemsa's stain or Leishman's stain and were observed under light microscope (Hund H500).

2.4. Identification and Counting of Blood Cells. Erythrocytes and their variations were identified following Hadji-Azimi et al. [25], Sood [26], and Thrall [27]. The leucocytes were identified as lymphocytes, monocytes, eosinophils, neutrophils, and basophils, following Hadji-Azimi et al. [25] and Thrall [27]. Slides were viewed in zigzag pattern, covering all parts of the blood smear, and all leucocytes were counted in each field of view until 100 cells were counted. Per blood smear, 150 fields of views were randomly selected to assess erythrocytes. Only field of views with even distribution of erythrocytes was used. For the morphometric analysis of erythrocytes, fifty cells per blood smears were measured. The size of erythrocytes and their nuclei was measured by an ocular micrometer which was standardized against a stage micrometer (ERMA, Japan). For morphometric analysis, the formula of Arserim and Mermer [28] was followed. Photographs of the leucocytes and erythrocytes were taken with the help of a Canon EOS 450 12.2-megapixel camera (EF-S 18–55 IS Kit) connected to Hund 500 WETZLAR microscope.

2.5. Statistical Analysis. The relationship between developmental stages of tadpoles and blood cell profiles was assessed by drawing scatter plots. The correlation coefficient “*r*” was calculated in each case by Karl Pearson’s method [29].

3. Results

3.1. Blood Cells Profile of the Tadpoles of *M. ornata*

3.1.1. Morphology of Erythrocytes. The erythrocytes in all stages of the tadpoles were found to be circular (Figure 2(a)), oval (Figure 2(b)), and elliptical in shape (Figure 2(c)). Nuclei of these three types of erythrocytes were round in shape and placed mostly in the center. However, few erythrocytes had eccentric nuclei pushed to the periphery (Figure 2(d)). In tadpoles of stages 37 to 42, the erythrocytes were either unusually larger in size or smaller than the normal erythrocytes (Figures 2(e) and 2(f)). Poikilocytosis (20–30%) was observed, during different developmental stages (stages 26 to 33). Few erythrocytes (0.1–0.5%) lacked distinct membrane (Figure 2(g)) and some (0.5–1%) showed irregular shapes (Figures 2(h), 2(i), and 2(j)). Several tear drop forms (5–7%) (also called dacrocytes) with tapering or slightly blunt ends (Figure 2(k)), and comma-shaped cells (Figure 2(l)) were noticed in tadpoles between stages 38 and 42. Some erythrocytes (1–2%) were found to lack nuclei (Figure 2(m)) while in others (0.5–1%) the nuclei were indistinct (Figure 2(n)). Several smaller dark bodies surrounding regular erythrocytes were found in tadpoles of stages 40–42 (Figure 2(o)). In several smears (stages 40 to 42), the erythrocytes were found to have vacuole like structures where the nuclei were pushed to the extreme periphery (Figure 2(p)). Dark patches were found inside some intact erythrocytes (Figure 2(q)). In other such cells, the membrane appeared to be disintegrated (Figure 2(r)).

Of all the variations in the shape of the erythrocytes, the most remarkable was the presence of several kinds of crenulated erythrocytes that had strong resemblance with echinocytes and acanthocytes as reported in mammalian peripheral smears. The crenulations showed a pattern of appearance as they were found in the peripheral smear of tadpoles between Gosner stages 37 and 45. Initially crenulations appeared as fine serrations in the erythrocytes of tadpoles of Gosner stages 37–40 resembling echinocytes (Figures 3(a) and 3(b)). Subsequently, highly crenulated forms resembling acanthocytes appeared on erythrocytes towards the climax stage (Gosner stages 41 to 45) (Figures 3(c), 3(d), and 3(e)). However, the maximum number of such elaborately spiked cells was seen in stage 44 tadpoles. These cells were absent in the froglets of stage 46. The crenulated cells had approximately 8–10 spiny projections distributed uniformly all over (Figures 3(c) and 3(d)) while few had developed 3–4 projections only at one side (Figure 3(e)). Moreover, several degenerating erythrocytes (Figure 3(f)) were recorded in the blood smears of the tadpoles of stages 42 to 44. In these stages, many erythrocytes were in the state of division (Figures 3(g), 3(h), 3(i), and 3(j)). Aggregation of erythrocytes was evident throughout developmental stages of the tadpoles (Figure 3(k)).

3.1.2. Morphology of Leucocytes. Leucocytes were of five different types, that is, lymphocytes, monocytes, eosinophils, neutrophils, and basophils. The lymphocytes, both large and small (Figures 4(a) and 4(b), resp.), were round in shape. Their nuclei were also rounded and occupied almost the entire cell leaving a narrow rim of light violet cytoplasm towards the periphery. Some lymphocytes showed irregular membrane (Figure 4(c)). Monocytes had eccentrically placed indented nuclei (Figure 4(d)). Eosinophils showed bilobed nuclei where the connections between the lobes were distinct (Figure 4(e)). Neutrophils (Figure 4(f)) with trilobed and tetralobed nuclei were also observed. Neutrophils with trilobed nuclei were the most common in tadpoles. Basophils were identified by the presence of dark violet stained granules over the nuclei as well as entire cells (Figure 4(g)).

3.1.3. Blood Thrombocytes. The blood thrombocytes were small in size and were found in clusters of 8 to 25 cells (Figure 4(h)).

3.1.4. Morphometry of Erythrocytes. The length (*L*) of erythrocytes ranged from $15.30 \pm 2.94 \mu\text{m}$ (Gosner stage 42) to $19.99 \pm 2.60 \mu\text{m}$ (Gosner stage 44) while the breadth (*B*) of the erythrocytes ranged from $15.30 \pm 2.24 \mu\text{m}$ and $15.30 \pm 3.64 \mu\text{m}$ (Gosner stages 36 and 43, resp.) to $18.93 \pm 2.11 \mu\text{m}$ (Gosner stage 30), respectively (Table 1). Similarly, the length (*L'*) and breadth (*B'*) of the nuclei ranged from $7.42 \pm 1.50 \mu\text{m}$ (Gosner stage 42) to $10.60 \pm 1.79 \mu\text{m}$ (Gosner stage 27) and $7.42 \pm 1.50 \mu\text{m}$ (Gosner stages 41 and 43) to $10.30 \pm 1.48 \mu\text{m}$ (Gosner stage 30), respectively (Table 1). Moreover, area occupied by the erythrocytes (*A*) ranged from $182.06 \pm 22.90 \mu\text{m}^2$ (Gosner stage 43) to $282.00 \pm 41.94 \mu\text{m}^2$ (Gosner stage 30) while the area of nuclei of the erythrocytes

FIGURE 2: Erythrocytes and its variations in the tadpoles of the ornate frog, *Microhyla ornata*. (a) Circular erythrocytes, (b) oval erythrocyte, (c) elliptical erythrocytes, (d) erythrocyte with eccentrically positioned nucleus, (e) large sized erythrocyte, (f) small sized erythrocyte, and (g) erythrocyte lacking a distinct membrane (stained in Leishman's stain). ((h), (i), (j)) Irregular shaped erythrocytes, (k) tear drop shaped erythrocyte (stained in Leishman's stain), (l) comma shaped erythrocyte (stained in Leishman's stain), (m) erythrocyte lacking nucleus, (n) erythrocyte lacking a distinct nucleus, (o) small dark bodies surrounding regular erythrocytes, (p) large erythrocyte with a vacuolated structure and nucleus pushed towards the periphery, (q) dark patches in intact erythrocytes, and (r) Disintegrated erythrocyte with dark patches. Scale bar = 10 μm .

(A') ranged from $45.05 \pm 12.86 \mu\text{m}^2$ (Gosner stage 42) to $83.97 \pm 23.28 \mu\text{m}^2$ (Gosner stage 30). The ratio of the area of the nuclei of erythrocytes to the area of the erythrocytes (A'/A) ranged from 0.21 ± 0.03 (Gosner stage 40) to 0.32 ± 0.03 (Gosner stage 28). The length to breadth (L/B) ratio of the erythrocytes ranged between 0.83 ± 0.11 (Gosner stage 45) and 1.08 ± 0.11 (Gosner stage 27 and Gosner stage 40), while the length to breadth ratio of the nuclei (L'/B') ranged

from 0.94 ± 0.06 (Gosner stage 32) to 1.16 ± 0.06 (Gosner stage 43) in the tadpoles of different developmental stages.

3.1.5. Differential Leucocyte Count. Lymphocytes were the most abundant cells amongst all leucocytes (Table 2). There was a surge in their percentage from 48.09 ± 1.2 (stage 46) to 95.88 ± 1.6 (stage 26). The percentage of monocytes ranged from 0 to 17.06 ± 0.9 . The highest percentage of

FIGURE 3: Different types of erythrocytes in the tadpoles of the ornate frog, *Microhyla ornata*. (a) Early stage of crenulated erythrocytes and (b) erythrocyte with increased size and distinct serrations (resembling echinocytes) ((c), (d)) Erythrocytes (resembling acanthocytes) with distinct spikes uniformly around the cell, (e) erythrocyte (resembling acanthocytes) having spikes on only one side, and (f) degenerating erythrocyte and ((g), (h), (i), (j)). Dividing erythrocytes. (k) Aggregation of erythrocytes. Scale bar = 10 μm .

monocytes was found in stage 42 tadpoles. Interestingly, their percentage remained high during metamorphic stages in comparison with the earlier stages. The percentage of eosinophils ranged from 0 to 16.13 ± 0.3 , and the highest percentage was observed during stage 46. However, their number was low during the early developmental stages. The neutrophils remained the second abundant leucocytes after lymphocytes. Their percentage ranged between 0 and 19.66 ± 0.9 . No neutrophils were observed during stage 29 while they were high during Gosner stage 46 (Table 2).

The highest percentage of basophils was recorded during stage 46 tadpoles. Their overall percentage was low in the peripheral blood smears of the tadpoles as their percentage fluctuated between 0 and 6.05 ± 0.2 .

3.2. Blood Cells Profile of Adult *Microhyla ornata*

3.2.1. Morphology of Erythrocytes. The erythrocytes of adult *M. ornata* were elliptical in shape (Figure 5(a)) while only

FIGURE 4: Leucocytes and thrombocytes in the tadpoles of the ornate frog, *Microhyla ornata*. (a) Large lymphocyte, (b) small lymphocyte, (c) lymphocyte with irregular margin (stained in Leishman's stain), (d) monocyte (stained in Leishman's stain), (e) eosinophil, (f) neutrophil, (g) basophil, and (h) thrombocytes. Scale bar = 10 μm .

few were found to be circular (Figure 5(b)) with centrally placed nuclei. Only a few tear drop shaped cells (Figure 5(c)), and comma shaped cells (Figure 5(d)) were found and other variations in shapes of erythrocytes were not recorded at all.

3.2.2. Morphology of Leucocytes. Both granulocytes and agranulocytes were found in the peripheral blood smear of adult frogs. The lymphocytes were of two types, large (Figure 5(e)) and small (Figure 5(f)). Their nuclei were large, centrally placed, and occupied majority of the area leaving a narrow rim of cytoplasm. Monocytes (Figure 5(g)) had eccentrically placed indented nuclei. Eosinophils were identified with the bilobed nuclei (Figure 5(h)). Neutrophils had multilobed nuclei. Usually 3 or 4 lobes were recorded (Figures 5(i) and 5(j), resp.). However, few cells had hypersegmented nuclei (Figures 5(k) and 5(l)). Several neutrophils had ring-like nucleus in which all lobes joined or folded to give a ring-like appearance (Figure 5(m)). Band neutrophils were also

recorded (Figure 5(n)). Basophils (Figure 5(o)) were darkly stained and smaller cells with heavy granule deposition.

3.2.3. Thrombocytes. Thrombocytes were nucleated and spindle shaped (Figure 5(p)).

3.2.4. Morphometry of Erythrocytes. The surface area covered by erythrocytes in adult males was found to be $254.39 \pm 40.82 \mu\text{m}^2$ while it was recorded to be $238.29 \pm 29.75 \mu\text{m}^2$ in adult females (Table 1). Therefore, the surface area occupied by erythrocytes was more in males than females. The mean length and breadth of erythrocytes were found to be $22.11 \pm 2.16 \mu\text{m}$ and $13.18 \pm 2.20 \mu\text{m}$, in adult males. In adult females their values were found to be $21.96 \pm 2.11 \mu\text{m}$ and $13.93 \pm 2.22 \mu\text{m}$, respectively. Similarly, the mean length and breadth of nuclei of erythrocytes in adult males were recorded to be $12.27 \pm 1.16 \mu\text{m}$ and $6.81 \pm 1.62 \mu\text{m}$, respectively, while

TABLE 1: Morphometry of erythrocytes during the larval development and in the adults of the ornate frog, *Microhyla ornata*.

Gosner	Stages Taylor and Kollros	STL	Size of cell			Size of nuclei				A'/A ± SD
			Length (L ± SD) (μm)	Breadth (B ± SD) (μm)	L/B ± SD	Area (A ± SD) (μm ²)	Length (L' ± SD) (μm)	Breadth (B' ± SD) (μm)	L'/B' ± SD	Area (A' ± SD) (μm ²)
26	I	1.5 ± 0.0	16.05 ± 2.22	17.27 ± 2.89	0.92 ± 0.11	218.45 ± 48.11	9.09 ± 1.65	9.39 ± 1.32	0.96 ± 0.06	68.10 ± 20.06
27	II	1.5 ± 0.0	19.24 ± 1.73	17.72 ± 1.98	1.08 ± 0.11	267.46 ± 36.32	10.60 ± 1.79	9.39 ± 1.63	1.12 ± 0.06	78.58 ± 21.62
28	III	1.7 ± 0.0	17.11 ± 2.46	16.51 ± 1.78	1.03 ± 0.11	221.67 ± 35.81	9.39 ± 1.32	9.69 ± 1.81	0.96 ± 0.06	71.36 ± 16.58
29	IV	1.7 ± 0.04	16.66 ± 2.24	16.81 ± 1.50	0.99 ± 0.11	219.50 ± 31.70	9.39 ± 1.32	9.24 ± 0.66	1.01 ± 0.06	68.06 ± 10.32
30	V	1.7 ± 0.04	19.08 ± 2.55	18.93 ± 2.11	1.00 ± 0.11	282.00 ± 41.94	10.30 ± 2.00	10.30 ± 1.4	1.00 ± 0.06	83.97 ± 23.28
31	VI	1.7 ± 0.12	18.78 ± 1.54	18.18 ± 1.35	1.03 ± 0.11	267.80 ± 26.14	9.24 ± 1.16	8.63 ± 1.73	1.07 ± 0.06	62.35 ± 13.53
32	VII	1.7 ± 0.09	15.45 ± 3.69	15.60 ± 3.07	0.99 ± 0.11	182.78 ± 29.76	7.72 ± 1.50	8.18 ± 1.68	0.94 ± 0.06	48.66 ± 10.40
33	VIII	1.8 ± 0.09	17.57 ± 1.81	16.36 ± 1.76	1.07 ± 0.11	224.58 ± 23.16	9.84 ± 1.31	9.24 ± 0.66	1.06 ± 0.06	71.36 ± 9.92
34	IX	1.9 ± 0.04	16.65 ± 1.51	17.57 ± 1.81	0.97 ± 0.11	229.96 ± 31.73	9.39 ± 0.90	9.39 ± 0.90	1.00 ± 0.06	69.90 ± 15.13
35	X	1.8 ± 0.0	15.75 ± 1.54	15.60 ± 1.98	1.00 ± 0.11	192.48 ± 26.42	7.72 ± 1.50	8.02 ± 1.44	0.96 ± 0.06	48.65 ± 13.19
36	XI	2.0 ± 0.0	15.75 ± 1.54	15.30 ± 2.24	1.02 ± 0.11	193.19 ± 33.63	9.24 ± 0.66	8.33 ± 1.31	1.10 ± 0.06	60.54 ± 11.02
37	XII	2.5 ± 0.0	16.65 ± 1.51	15.90 ± 1.31	1.04 ± 0.11	208.68 ± 30.02	9.09 ± 1.35	8.02 ± 1.44	1.13 ± 0.06	57.66 ± 14.94
38	XIII	2.4 ± 0.16	16.36 ± 1.76	16.21 ± 1.73	1.00 ± 0.11	207.61 ± 27.97	8.93 ± 1.50	8.18 ± 1.38	1.09 ± 0.06	57.66 ± 14.94
39	XIV	2.4 ± 0.18	16.66 ± 2.24	16.66 ± 1.51	1.00 ± 0.11	216.30 ± 22.43	8.78 ± 1.63	8.18 ± 1.38	1.07 ± 0.06	56.94 ± 15.93
40	XV-XVII	2.2 ± 0.2	18.02 ± 1.78	16.66 ± 1.79	1.08 ± 0.11	235.74 ± 33.12	8.33 ± 1.31	7.72 ± 1.78	1.07 ± 0.06	51.53 ± 16.94
41	XVIII-XIX	2.3 ± 0.04	17.87 ± 1.63	16.51 ± 2.02	1.08 ± 0.11	231.03 ± 30.33	8.18 ± 1.38	7.42 ± 1.50	1.10 ± 0.06	47.93 ± 13.53
42	XX	2.2 ± 0.0	15.30 ± 2.94	17.26 ± 3.96	0.88 ± 0.11	195.40 ± 42.44	7.42 ± 1.50	7.72 ± 1.50	0.96 ± 0.06	45.05 ± 12.86
43	XXI	1.4 ± 0.04	15.90 ± 3.81	15.30 ± 3.64	1.03 ± 0.11	182.06 ± 22.90	8.63 ± 1.11	7.42 ± 1.50	1.16 ± 0.06	50.81 ± 13.56
44	XXII	1.2 ± 0.0	19.99 ± 2.60	13.78 ± 3.10	1.45 ± 0.11	211.27 ± 24.24	8.93 ± 0.66	8.18 ± 1.38	1.09 ± 0.06	57.66 ± 11.38
45	XXIII-XXIV	0.7 ± 0.08	15.45 ± 2.69	18.48 ± 2.69	0.83 ± 0.11	219.56 ± 21.44	8.48 ± 1.21	8.78 ± 0.90	0.96 ± 0.06	58.38 ± 9.89
46	XXV	0.6 ± 0.0	16.05 ± 1.38	16.21 ± 1.73	0.99 ± 0.11	204.02 ± 24.36	8.18 ± 1.38	7.59 ± 1.51	1.07 ± 0.06	48.65 ± 12.86
Correlation coefficient (r)			-0.208	-0.348	+0.124	-0.438	-0.591	-0.758	+0.296	-0.713
Sex (no. of adult frogs used)			Average SVL (cm) ± SD	Size of cell			Size of nuclei			
			Length (L ± SD) (μm)	Breadth (B ± SD) (μm)	L/B ± SD	Area (A ± SD) (μm ²)	Length (L' ± SD) (μm)	Breadth (B' ± SD) (μm)	L'/B' ± SD	Area (A' ± SD) (μm ²)
Males (7)			10.12 ± 0.84	1.8 ± 0.31	22.11 ± 2.16	13.18 ± 2.20	1.73 ± 0.34	12.27 ± 1.16	6.81 ± 1.62	65.96 ± 18.13
Females (7)			11.86 ± 0.73	1.9 ± 0.12	21.96 ± 2.11	13.93 ± 2.22	1.62 ± 0.32	12.42 ± 1.63	7.72 ± 1.78	76.43 ± 23.51

STL: snout to tail tip length; SVL: snout to vent length; SD: standard deviation; μm: micrometer.

FIGURE 5: Blood cells of the adult ornate frog, *Microhyla ornata*. (a) Elliptical erythrocytes, (b) circular erythrocyte, (c) tear drop shaped erythrocyte, (d) comma shaped erythrocyte, (e) large lymphocyte, (f) small lymphocyte, (g) monocyte, (h) eosinophil, (i) neutrophil with 3 lobed nuclei, (j) neutrophil with 4 lobed nuclei and ((k), (l)) neutrophils with hypersegmented nuclei, (m) neutrophil with folded nuclei forming ring, (n) band neutrophil, (o) basophil, and (p) thrombocyte. Scale bar = 10 μm .

their values in adult females were found to $12.42 \pm 1.63 \mu\text{m}$ and $7.72 \pm 1.78 \mu\text{m}$, respectively. The ratio between the area of nuclei of the erythrocytes to the area of the erythrocytes (A'/A) was found to be 0.26 ± 0.08 in male frogs while it was 0.31 ± 0.08 in the females. The length to breadth ratio of the erythrocytes (L/B) was found to be 1.73 ± 0.34 and 1.62 ± 0.32 in the male and female frogs, respectively. Similarly, the length to breadth ratio of nuclei of the erythrocytes (L'/B') in adult males and females was recorded to be 1.86 ± 0.32 and 1.66 ± 0.31 , respectively.

3.2.5. Differential Leucocyte Count. The mean lymphocytes and monocytes percentage in adult males were found to be 50.42 ± 5.2 and 6.24 ± 1.2 , respectively. In adult females the percentage was found to be 51.28 ± 6.3 and 5.28 ± 1.8 , respectively. In adult males, mean eosinophil percentage was 10.21 ± 1.9 while it was 9.32 ± 1.9 in adult females (Table 2). Mean neutrophil percentage in adult males was 30.41 ± 6.8 and in adult females it was found to be 31.21 ± 10.2 . The mean basophil percentage in adult males was 2.72 ± 1.0 while it was 2.91 ± 0.7 in adult females. In the adult frogs, percentage of

TABLE 2: Percentage of leucocytes during larval development and in the adults of the ornate frog, *Microhyla ornata*.

Stages		Lymphocytes $\pm$ SD	Monocytes $\pm$ SD	Eosinophils $\pm$ SD	Neutrophils $\pm$ SD	Basophils $\pm$ SD
Gosner stage	Taylor and Kollros stage					
26	I	95.88 $\pm$ 1.6	1.17 $\pm$ 1.6	0.00 $\pm$ 0.0	2.95 $\pm$ 0.0	0.00 $\pm$ 0.0
27	II	92.86 $\pm$ 2.5	4.28 $\pm$ 1.5	0.00 $\pm$ 0.0	2.86 $\pm$ 1.5	0.00 $\pm$ 0.0
28	III	87.87 $\pm$ 2.1	3.05 $\pm$ 2.1	0.60 $\pm$ 1.3	3.63 $\pm$ 1.3	4.85 $\pm$ 1.6
29	IV	87.78 $\pm$ 2.4	1.12 $\pm$ 2.4	5.55 $\pm$ 3.9	0.00 $\pm$ 0.0	5.55 $\pm$ 0.0
30	V	91.44 $\pm$ 1.0	4.28 $\pm$ 1.0	1.44 $\pm$ 0.0	1.42 $\pm$ 0.0	1.42 $\pm$ 1.0
31	VI	87.75 $\pm$ 3.5	3.18 $\pm$ 0.1	0.00 $\pm$ 0.0	5.85 $\pm$ 2.7	3.22 $\pm$ 2.2
32	VII	93.17 $\pm$ 0.5	1.06 $\pm$ 0.0	1.26 $\pm$ 0.4	3.23 $\pm$ 0.0	1.28 $\pm$ 0.4
33	VIII	87.10 $\pm$ 2.2	0.00 $\pm$ 0.0	0.00 $\pm$ 0.0	8.39 $\pm$ 1.7	4.51 $\pm$ 1.7
34	IX	87.09 $\pm$ 0.4	2.30 $\pm$ 0.1	1.25 $\pm$ 1.7	5.3 $\pm$ 2.0	4.06 $\pm$ 0.0
35	X	85.85 $\pm$ 1.6	3.56 $\pm$ 0.8	0.59 $\pm$ 0.7	5.29 $\pm$ 1.7	4.71 $\pm$ 1.7
36	XI	94.43 $\pm$ 0.7	1.87 $\pm$ 0.7	1.43 $\pm$ 0.0	2.27 $\pm$ 0.3	0.00 $\pm$ 0.0
37	XII	86.45 $\pm$ 0.6	2.17 $\pm$ 0.4	2.33 $\pm$ 0.5	2.23 $\pm$ 0.8	5.82 $\pm$ 1.3
38	XIII	93.71 $\pm$ 1.4	0.00 $\pm$ 0.0	0.00 $\pm$ 0.0	2.34 $\pm$ 0.5	3.95 $\pm$ 0.8
39	XIV	82.13 $\pm$ 1.1	4.21 $\pm$ 0.1	7.76 $\pm$ 1.1	2.76 $\pm$ 0.7	3.14 $\pm$ 0.2
40	XV-XVII	88.67 $\pm$ 1.3	3.82 $\pm$ 1.2	1.86 $\pm$ 0.1	3.77 $\pm$ 0.0	1.88 $\pm$ 0.0
41	XVIII-XIX	82.38 $\pm$ 1.3	3.92 $\pm$ 0.0	3.82 $\pm$ 0.2	7.84 $\pm$ 0.0	2.04 $\pm$ 1.5
42	XX	61.09 $\pm$ 0.7	17.06 $\pm$ 0.9	15.08 $\pm$ 0.2	5.24 $\pm$ 0.3	1.53 $\pm$ 0.4
43	XXI	58.80 $\pm$ 1.1	11.08 $\pm$ 0.6	14.77 $\pm$ 0.1	12.09 $\pm$ 0.5	3.26 $\pm$ 0.8
44	XXII	57.26 $\pm$ 0.1	9.43 $\pm$ 0.3	15.06 $\pm$ 0.2	16.19 $\pm$ 0.1	2.06 $\pm$ 0.3
45	XXIII-XXIV	58.06 $\pm$ 0.1	9.17 $\pm$ 0.1	12.84 $\pm$ 0.1	15.01 $\pm$ 0.3	4.92 $\pm$ 0.4
46	XXV	58.09 $\pm$ 1.2	10.07 $\pm$ 0.1	16.13 $\pm$ 0.3	19.66 $\pm$ 0.9	6.05 $\pm$ 0.2
Correlation coefficient (r)		-0.801	+0.654	+0.779	+0.719	+0.249
Adult Frogs	Males	50.42 $\pm$ 5.2	6.24 $\pm$ 1.2	10.21 $\pm$ 1.9	30.41 $\pm$ 6.8	2.72 $\pm$ 1.0
	Females	51.28 $\pm$ 6.3	5.28 $\pm$ 1.8	9.32 $\pm$ 1.9	31.21 $\pm$ 10.2	2.91 $\pm$ 0.7

SD: standard deviation.

monocytes and eosinophils was higher in males than females, but percentages of lymphocytes, basophils, and neutrophils were higher in females than males (Table 2).

3.3. Statistical Analysis. In the tadpoles, a negative correlation was observed between different stages with respect to length ($r = -0.208$), breadth ($r = -0.348$), and area ($r = -0.438$) of the erythrocytes (Figure 6). Similar negative correlation was observed for length ($r = -0.591$), breadth ($r = -0.758$), and area ($r = -0.713$) of nuclei of the erythrocytes (Figure 7). However, the aspect ratio of the erythrocytes (L/B) and their nuclei (L'/B') were positively correlated $r = +0.124$ in Figure 6(c) and $r = +0.296$ in Figure 7(c), respectively. The A'/A ratio was found to be negatively correlated ($r = -0.591$) (Figure 7). The monocytes ($r = +0.654$), eosinophils ($r = +0.779$), neutrophils ($r = +0.719$), and basophils ($r = +0.249$) showed a positive correlation, whereas lymphocytes ($r = -0.801$) showed a negative correlation with different stages during development (Figure 8).

4. Discussion

The erythrocytes of the tadpoles of *M. ornata* were observed to be round, oval, or elliptical in shape (Figures 2(a), 2(b), and 2(c)). This observation is similar to previous studies

on larval amphibians which suggest presence of two general forms of erythrocytes, larval and adult forms [30–32]. The larval form is large and elongated while the adult form is smaller and rounder. Nucleus, a characteristic feature of amphibian erythrocytes, was found to be placed at the center of the erythrocytes in almost all peripheral smears. However, few variations were also recorded where nuclei were either eccentrically positioned or pushed to the periphery (Figure 2(d)). In tadpoles of stages 37 to 42, several erythrocytes were noticed to be either unusually larger in size or smaller than the normal erythrocytes (Figures 2(e) and 2(f)). Few erythrocytes lacked a distinct membrane (Figure 2(g)) while several others had irregular appearance (Figures 2(h), 2(i), and 2(j)). Poikilocytosis was noticed in the peripheral blood smears of the tadpoles during the early stages in patches only. Abnormal shape of the erythrocytes included several tear drop forms (Figure 2(k)) and comma-shaped erythrocytes (Figure 2(l)). Some erythrocytes were found to lack nuclei (Figure 2(m)) while in others the nuclei were indistinct (Figure 2(n)). Similar large sized erythrocytes and erythrocytes lacking nucleus (senile erythrocytes) have been reported in tadpoles of *Polypedates teraiensis* [23]. Such senile erythrocytes have also been reported during metamorphosis in other anurans [33]. Several smaller dark bodies surrounding regular erythrocytes (Figure 2(o)) were observed in the blood smears of the tadpoles of stages 40 to 42. In

FIGURE 6: Correlation between different morphometric values of erythrocytes with respect to the developmental stages of the tadpoles of the ornate frog, *Microhylla ornata*.

these stages few erythrocytes appeared large in size having vacuolated structures that pushed the nuclei to the periphery making them look inconspicuous (Figure 2(p)). Moreover, dark patches were found inside some intact erythrocytes (Figure 2(q)). In other such cells, the membrane appeared to be disintegrated (Figure 2(r)). Interestingly, a remarkable degree of crenulated erythrocytes was also noticed (Figures 3(a), 3(b), 3(c), 3(d), and 3(e)) towards the late prometamorphic and metamorphic stages. Crenulations have been previously reported in red blood cells of *Rana pipiens* after forelimb emergence [30]. These cells resembling echinocytes and acanthocytes of mammalian red blood cells have also been reported earlier in thyroid-treated tadpoles of *Rana catesbeiana* where cells were irregular with many cytoplasmic projections [34] and in tadpoles of *P. teraiensis* [23]. Such cells have been described to be present during anemic conditions [34, 35], and the ectothermic animals are capable to withstand anemic conditions for a long period without mortality [23, 36]. Erythrocytes which had undergone degeneration were also observed (Figure 3(f)).

The size and shape of erythrocyte give an indication of the surface available for the exchange of gases in respiratory functions [37]. Morphometric measurement of the erythrocytes (Table 1) confirmed a negative correlation existing between the developmental stages with respect to the size of the erythrocytes and their nuclei. However, their aspect ratio (length/breadth) was found to be positive. A gradual decline in the area occupied by the erythrocytes was observed

in the tadpoles with progress in development as a negative correlation existed between the developmental stages with respect to the area of the erythrocytes and their nuclei. The smallest erythrocytes were present in the metamorphosed froglets. Broyles [38], Duellman, and Trueb [39] have described replacement of larger larval erythrocytes by smaller adult erythrocytes during metamorphosis in several anurans. Several cells were found to be in different stages of division as nuclear division was distinct (Figures 3(g), 3(h), 3(i), and 3(j)). An earlier report of increase in erythropoietic activity during metamorphosis has also been reported in *R. catesbeiana* [40]. Another interesting observation was aggregation of several erythrocytes during different developmental stages (Figure 3(k)).

Several trends with respect to the percentage of leucocytes during different developmental stages appeared consistent with the earlier observations [3, 6, 7, 23]. Lymphocytes were found to be most abundant during the growth phase of larval development. Their percentage decreased towards end of metamorphosis (Table 2). Davis has reported 70% of lymphocytes in the tadpoles of stages 30 to 33 in *R. catesbeiana* which decreased with onset of metamorphosis. The trend in monocyte which is a phagocytic cell remained at par with the earlier observations of Davis [3]. Their number increased significantly during the metamorphic stages. The higher number of monocytes has been correlated with the increased cellular debris left over from the tissue lysis during

FIGURE 7: Correlation between different morphometric values of erythrocytes with respect to the developmental stages of the tadpoles of the ornate frog, *Microhyla ornata*.

remodeling of larval structures [3], and a positive correlation was observed with developmental stages.

However, an unusual spike of neutrophils was observed during the metamorphic climax in the present study unlike the earlier reports on *Rana catesbeiana* [3] and *P. teraiensis* [23] where these cells were least abundant during the metamorphic period. Neutrophils remained significantly positive with respect to the developmental stages. The rise in the level of neutrophil in this species is suggested to be related to physiological condition. Eosinophils remained positively

correlated with the developmental stages of the tadpoles. Since these cells are known to produce a variety of chemical substances [41] and respond to tissue injury [42], an elevation in the level of eosinophils in the present study is suggested to act to modulate the process of lysis of tissue during metamorphosis. The percentage of basophils fluctuated during development. However, a positive correlation was observed between percentage of basophils and developmental stages, but the correlation was not significant. Increase in the basophil level has been reported in tadpoles of *R. catesbeiana*

FIGURE 8: Correlation between the percentages of different types of leucocytes with respect to the developmental stages of the tadpoles of the ornate frog, *Microhyla ornata*.

[3] and *Polypedates teraiensis* [23]. Thrombocytes were found to be in clusters or stacked with each other during different developmental stages.

The erythrocytes of adult *Microhyla ornata* were ellipsoidal in shape with centrally placed nuclei, and only a few rounded erythrocytes were observed. The characteristic shape of anuran erythrocyte has been reported to be ellipsoidal [43]. Tok et al. [44] have reported morphologically similar erythrocytes among various species of anurans. Ellipsoidal erythrocytes have also been reported in the blood smear of *Polypedates maculatus* [23]. Erythrocytes being the most important carrier of oxygen and carbon dioxide, it has been suggested that an elliptical body is more efficient than a spherical one of the same volume as far as greater rate of

exchange is concerned [37]. Only a few tear drop shaped cells and comma-shaped cells were found in the peripheral smear. However, other variations in shapes of erythrocytes were not recorded in the adults as found in their larval counterparts. In the present study the surface area covered by erythrocytes was found to be greater in males than in females (Table 1). Similar reports of higher surface area of erythrocytes in males ($243.15 \pm 7.841 \mu\text{m}^2$) in comparison with females ($210.58 \pm 38.279 \mu\text{m}^2$) have been reported in adult frogs of *P. maculatus* [23]. In the present study, the mean breadth of the erythrocytes and their nuclei was found to be slightly higher in females than of males (Table 1). Arserim and Mermer [28] have reported larger erythrocyte length and breadth in cases of females ($23.03 \mu\text{m}$ and $14.59 \mu\text{m}$

resp.) than males ($22.32\ \mu\text{m}$ and $13.65\ \mu\text{m}$, resp.) in *Rana macrocnemis*. The size of erythrocytes of adult *M. ornata* was found to be higher in comparison with the erythrocytes length ($15.4\ \mu\text{m} \pm 1.04$) and breadth ($11.47\ \mu\text{m} \pm 0.82$) of the balloon frog *Glyphoglossus molossus* belonging to family microhylidae [45].

Leucocyte differential in the adult frogs showed percentage of monocytes and eosinophils to be slightly higher in males than females, but percentages of lymphocytes, basophils, and neutrophils were higher in females. Davis and Durso [4] have reported lymphocytes and neutrophils to be the most commonly seen cell types within amphibians. They have also reported the average of the white blood cells for anurans as 52.6 for lymphocytes, 29.1 for neutrophils, 7.0 for eosinophils, 7.5 for basophils, and 4.0 for monocytes. Comparable percentages of lymphocytes and neutrophils were observed in the present study. But, the percentages of basophils remained low while the percentages of eosinophils and monocytes remained higher. Thrombocytes in the present study were nucleated and spindle shaped. Earlier reports suggest amphibian thrombocytes to be nucleated and having spindle appearance [13, 28].

Thus, our study provides the baseline information on blood cell profile of tadpoles and adults of the ornate frog, *Microhyla ornata*. Moreover, it describes the changes that take place in shape, size and number of blood cells during metamorphosis necessary for the aquatic tadpoles to adapt to a terrestrial environment as a froglet.

Acknowledgments

The authors would like to thank the Head of the P. G. Department of Zoology, Utkal University for providing necessary facilities. Madhusmita Das would like to thank UGC for a Research Fellowship (RFSMS). The authors would like to thank DST, the Government of India for financial assistance under PURSE Grant to the P. G. Department of Zoology, Utkal University.

References

- [1] J. Wojtaszek and A. Adamowicz, "Haematology of the fire-bellied toad, *Bombina bombina* L.," *Comparative Clinical Pathology*, vol. 12, no. 3, pp. 129–134, 2003.
- [2] S. F. Gilbert, *Developmental Biology*, Sinauer Associates, Sunderland, Mass, U.S.A, 2003.
- [3] A. K. Davis, "Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*)," *Comparative Clinical Pathology*, vol. 18, no. 2, pp. 181–186, 2009.
- [4] A. K. Davis and A. M. Durso, "White blood cell differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians," *Herpetologica*, vol. 65, no. 3, pp. 260–267, 2009.
- [5] M. K. Atatür, H. Arikan, and I. E. Çevik, "Erythrocyte sizes of some anurans from Turkey," *Turkish Journal of Zoology*, vol. 23, pp. 111–114, 1999.
- [6] H. E. Jordan and C. C. Speidel, "Leukocytes in relation to the mechanism of thyroid-accelerated metamorphosis in the larval frog," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 20, pp. 380–383, 1922.
- [7] H. E. Jordan and C. C. Speidel, "The behavior of the leucocytes during coincident regeneration and thyroid-induced metamorphosis in the frog larva, with a consideration of growth factors," *The Journal of Experimental Medicine*, vol. 40, pp. 1–11, 1924.
- [8] J. M. Kiesecker, "Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature?" *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 15, pp. 9900–9904, 2002.
- [9] S. Barni, E. Boncompagni, A. Grosso et al., "Evaluation of *Rana snk esculenta* blood cell response to chemical stressors in the environment during the larval and adult phases," *Aquatic Toxicology*, vol. 81, no. 1, pp. 45–54, 2007.
- [10] H. M. Smith, "Cell size and metabolic activity in amphibian," *The Biological Bulletin*, vol. 48, no. 5, pp. 347–378, 1925.
- [11] F. J. Vernberg, "Hematological studies in salamanders in relation to their ecology," *Herpetologica*, vol. 11, no. 2, pp. 129–133, 1955.
- [12] K. Mitsuru, "Relationships between number, size and shape of red blood cells in amphibians," *Comparative Biochemistry and Physiology A*, vol. 69, no. 4, pp. 771–775, 1981.
- [13] M. C. Allender and M. M. Fry, "Amphibian hematology," *Veterinary Clinics of North America*, vol. 11, no. 3, pp. 463–480, 2008.
- [14] P. Mohanty-Hejmadi, S. K. Dutta, and I. Khan, "Life history of the Indian frogs. III. The ornate frog, *Microhyla ornata*," *Journal of the Zoological Society of India*, vol. 32, no. 1-2, pp. 43–48, 1980.
- [15] J. Rao and M. N. Madhyastha, "Toxicities of some heavy metals to the tadpoles of frog, *Microhyla ornata* (Dumeril & Bibron)," *Toxicology Letters*, vol. 36, no. 2, pp. 205–208, 1987.
- [16] M. Kuramoto and S. H. Joshy, "Morphological and acoustic comparisons of *Microhyla ornata*, *M. fissipes*, and *M. okinavenensis* (Anura: Microhylidae)," *Current Herpetology*, vol. 25, no. 1, pp. 15–27, 2006.
- [17] M. Matsui, H. Ito, T. Shimada et al., "Taxonomic relationships within the pan-oriental narrow-mouth toad *Microhyla ornata* revealed by mtDNA analysis (Amphibia, Anura, Microhylidae)," *Zoological Science*, vol. 22, no. 4, pp. 489–495, 2005.
- [18] K. Joseph and T. G. Baby, "Changes in polyamine contents during development of the frog *Microhyla ornata*," *Development Growth and Differentiation*, vol. 32, no. 3, pp. 329–334, 1990.
- [19] P. Mohanty-Hejmadi, "Care and management of amphibian embryos," *Prakruti: Utkal University Journal*, vol. 11, pp. 81–87, 1977.
- [20] K. L. Gosner, "A simplified table for staging anuran embryos and larvae," *Herpetologica*, vol. 16, pp. 183–190, 1960.
- [21] A. C. Taylor and J. J. Kollros, "Stages in the normal development of *Rana pipiens* larvae," *The Anatomical Record*, vol. 94, no. 1, pp. 7–23, 1946.
- [22] W. McDiarmid and R. Altig, *Tadpoles: The Biology of Anuran Larvae*, The University of Chicago Press, Chicago, Ill, USA, 2000.
- [23] M. Das and P. K. Mahapatra, "Blood cell profiles of the tadpoles of the Dubois's tree frog, *Polypedates teraiensis* Dubois, 1986 (Anura: Rhacophoridae)," *The Scientific World Journal*, vol. 2012, Article ID 701746, 11 pages, 2012.
- [24] K. N. Wright, "Amphibian hematology," in *Amphibian Medicine and Captive Husbandry*, K. N. Wright and B. R. Whitaker, Eds., pp. 129–146, Krieger, Malabar, Fla, USA, 1st edition, 2001.

- [25] I. Hadji-Azimi, V. Coosemans, and C. Canicatti, "Atlas of adult *Xenopus laevis laevis* hematology," *Developmental and Comparative Immunology*, vol. 11, no. 4, pp. 807–874, 1987.
- [26] R. Sood, *Haematology for Students and Practitioners*, Jaypee Brothers, New Delhi, India, 1996.
- [27] M. A. Thrall, "Hematology of amphibians," in *Veterinary Hematology and Clinical Chemistry: Test and Clinical Test Presentations*, M. A. Thrall, D. C. Baker, and E. D. Lassen, Eds., Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2004.
- [28] S. K. Arserim and A. Mermer, "Hematology of the Uludağ frog, *Rana macrocnemis* Boulenger 1885 in Uludağ National Park (Bursa, Turkey)," *European Union Journal of Fisheries and Aquatic Sciences*, vol. 25, no. 1, pp. 39–46, 2008.
- [29] R. G. D. Steel and J. N. Torrie, *Principles and Procedures of Statistics*, McGraw Hill, London, UK, 1980.
- [30] J. G. Hollyfield, "Erythrocyte replacement at metamorphosis in the frog, *Rana pipiens*," *Journal of Morphology*, vol. 119, no. 1, pp. 1–6, 1966.
- [31] J. Benbassat, "Erythroid cell development during natural amphibian metamorphosis," *Developmental Biology*, vol. 21, no. 4, pp. 557–583, 1970.
- [32] R. H. Broyles, G. M. Johnson, P. B. Maples, and G. R. Kindell, "Two erythropoietic microenvironments and two larval red cell lines in bullfrog tadpoles," *Developmental Biology*, vol. 81, no. 2, pp. 299–314, 1981.
- [33] C. C. Speidel, "Bile pigment production and erythrocyte destruction in thyroid-treated amphibian larvae," *The Journal of Experimental Medicine*, vol. 63, pp. 703–712, 1926.
- [34] G. L. Vankin, E. M. Brandt, and W. DeWitt, "Ultrastructural studies on red blood cells from thyroxin-treated *Rana catesbeiana* tadpoles," *The Journal of Cell Biology*, vol. 47, no. 3, pp. 767–772, 1970.
- [35] College of American Pathologists, "Blood cell identification," *Hematology and Clinical Microscopy Glossary*, pp. 3–21, 2010.
- [36] M. E. Feder and W. W. Burggren, *Environmental Physiology of the Amphibians*, University of Chicago Press, 1st edition, 1992.
- [37] F. A. Hartman and M. A. Lessler, "Erythrocyte measurements in fishes amphibians and reptiles," *The Biological Bulletin*, vol. 126, no. 1, pp. 83–88, 1964.
- [38] R. H. Broyles, "Changes in the blood during amphibian metamorphosis," in *Metamorphosis, a Problem in Developmental Biology*, L. I. Gilbert and E. Frieden, Eds., pp. 461–490, Plenum Press, New York, NY, USA, 2nd edition, 1981.
- [39] W. L. Duellleman and L. Trueb, *Biology of Amphibians*, McGraw Hill, New York, NY, USA, 1986.
- [40] G. M. Maniatis and V. M. Ingram, "Erythropoiesis during amphibian metamorphosis: I. site of maturation of erythrocytes in *Rana catesbeiana*," *Journal of Cell Biology*, vol. 49, no. 2, pp. 372–379, 1971.
- [41] M. E. Rothenberg and S. P. Hogan, "The eosinophil," *Annual Review of Immunology*, vol. 24, pp. 147–174, 2006.
- [42] D. J. Adamko, S. O. Odemuyiwa, D. Vethanayagam, and R. Moqbel, "The rise of the phoenix: the expanding role of the eosinophil in health and disease," *Allergy*, vol. 60, no. 1, pp. 13–22, 2005.
- [43] B. B. Mahapatra, M. Das, S. K. Dutta, and P. K. Mahapatra, "Hematology of Indian rhacophorid tree frog *Polypedates maculatus* Gray, 1833 (Anura: Rhacophoridae)," *Comparative Clinical Pathology*, vol. 21, no. 4, pp. 453–460, 2012.
- [44] C. V. Tok, M. Tosunoğlu, D. Ayaz, K. Çiçek, and Ç. Gül, "Hematology of the lycian salamander, *lyciasalamandra fazilae*," *North-Western Journal of Zoology*, vol. 5, no. 2, pp. 321–329, 2009.
- [45] S. Ponsen, N.-A. Narkkong, S. Pamok, K. Sappaso, and W. Aengwanich, "Hematological values and morphological observation of blood cells in balloon frog, *Glyphoglossus molossus*," *Journal of Microscopy Society of Thailand*, vol. 22, pp. 71–75, 2008.