

SHORT COMMUNICATION

Hematological parameters and morphological characteristics of blood cells in turtle and tortoise species within captivity in Sri Lanka

Nadeesha H. N. Dilrukshi¹, Anura P. Jayasooriya^{2,3}, Gamika A. Prathapasinghe¹

¹Department of Livestock and Avian Sciences, Faculty of Livestock, Fisheries and Nutrition, Wayamba University of Sri Lanka, Makandura, Sri Lanka

²Department of Basic Veterinary Sciences, School of Veterinary Medicine, Faculty of Medical Sciences, University of the West Indies, Trinidad and Tobago, West Indies

³Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine Animal Science, University of Peradeniya, Peradeniya, Sri Lanka

ABSTRACT

Objective: This study was conducted to determine hematological reference ranges and morphological characteristics blood cells in healthy captive Sri Lankan fresh water turtle and tortoise species.

Materials and Methods: Following turtle species, namely, *Melanochelys trijuga parkeri* ($n = 06$), *M. trijuga thermalis* ($n = 06$), and *Lissemys punctata punctata* ($n = 06$) and a tortoise species *Geochelone elegans* ($n = 06$) were evaluated. Blood smears were stained using Leishman–Gram staining protocol. The differential white blood cell counts and morphology were assessed using the standard protocols.

Results: Both red blood cells and their nuclei were irregular in *M. trijuga parkeri*, whereas *M. trijuga thermalis* had oval-shaped and the nuclei were irregular in shape. *L. punctata punctata* and *G. elegans* had oval-shaped red blood cells and their nuclei were round in shape. In terms of differential counts, heterophils were the most abundant leukocyte type in all the species. *Melanochelys trijuga parkeri* had the highest heterophils value of 55% and the lowest value of 48.5% was recorded with *G. elegans*. Lymphocytes count was significantly high in *G. elegans*. Characteristic morphological features were observed in different leukocytes. In terms of thrombocytes, *M. trijuga thermalis* and *L. punctata punctata* had single platelets, whereas *M. trijuga parkeri* and *G. elegans* had platelets as clumps. Thrombocytes are ellipsoidal with centrally located dark-stained nuclei and their cytoplasm is clear.

Conclusion: The findings in this study can be used as the reference values in the assessment of health of the above species and also will be useful in future hematological studies related to these species.

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Introduction

Sri Lanka is an island with a vast variety of biologically diverse natural eco-systems and habitats. Amphibians and reptiles in terrestrial and freshwater eco-systems play vital roles as a prey or predator [4]. In Sri Lanka, testudines comprised five (05) marine turtle species and four (04) fresh water turtle species, including Black Turtle (*Melanochelys trijuga thermalis*), Parker's Black turtle (*Melanochelys trijuga parkeri*), Flap-Shell Turtle (*Lissemys punctata*), and Red-Eared Slider (*Trachemys scripta*). The Red-eared slider is known as an alien invasive species [5]. Of these, one sub-species, Parker's black turtle *M. trijuga parkeri*, is considered as an endemic taxon with few scattered records. It differs from the hard-shelled terrapin or the Black turtle (*M. trijuga thermalis*), in its pigmentation pattern on head and neck area. The *M. trijuga thermalis* is common and is distributed throughout the country except at mountain areas. The Soft-Shelled Terrapin or

punctata), and Red-Eared Slider (*Trachemys scripta*). The Red-eared slider is known as an alien invasive species [5]. Of these, one sub-species, Parker's black turtle *M. trijuga parkeri*, is considered as an endemic taxon with few scattered records. It differs from the hard-shelled terrapin or the Black turtle (*M. trijuga thermalis*), in its pigmentation pattern on head and neck area. The *M. trijuga thermalis* is common and is distributed throughout the country except at mountain areas. The Soft-Shelled Terrapin or

Correspondence Gamika A. Prathapasinghe ✉ gamika@gmail.com 🏢 Department of Livestock and Avian Sciences, Faculty of Livestock, Fisheries and Nutrition, Wayamba University of Sri Lanka, Makandura, Sri Lanka.

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Flap-shelled turtle (*L. punctata punctata*) is found in the low country. The Star tortoise (*G. elegans*) is the only land tortoise, which is commonly found in dry regions of the country [7] (Fig. 1).

Exploitation of natural habitat, human consumption, pettrade, depletion of prey species, and ingestion of synthetic materials are the main causes for turtle population reduction in Sri Lanka [6,10]. In 1999, IUCN has published a list of threatened species, which included *M. trijuga parkeri*, *L. punctata punctata*, and *G. elegans* species living in Sri Lanka in it. Furthermore, 10 reptile species, including *M. trijuga parkeri* have been recognized as endemic species and they are listed under the global red list published by IUCN in 2002.

A global action plan for conservation of tortoises and freshwater turtles has been implemented with some initiatives of captive breeding and management programs, promoting research activities, trade monitoring, and sustainable harvest programs throughout the world [8]. Sound

management and conservation of these species requires established scientific data, population dynamics, and positive community attitudes toward these aspects [11]. Interestingly, health condition is one of the most important parameter that should be considered in chelonian conservation and management [18]. Thus, knowledge related to normal reference ranges of hematological and physiological parameters are critically important to diagnose diseases, evaluate the health and nutritional status of tortoise populations and assess the impact of threatening factors [13]. Due to the different criteria used to identify blood cells and uncertain cellular lineages resulted in inconsistent classification of blood cells in reptiles [17]. However, it is difficult to implement conservation and management strategies aiming Sri Lankan species due to the scarcity of reliable data. Thus, the current study was conducted to determine the hematological parameters and morphological characteristics of different blood cells of aforementioned clinically healthy turtle and tortoise species within captivity in Sri Lanka.

Materials and Methods

Ethical statement

The research work was conducted according the guidance stipulated by Animal Ethics Committee of Wayamba University of Sri Lanka (Application No: 201509AI04, approval was granted on 22 September 2015). Though all these animal species were kept under captivity, they were provided with a suitable natural environment to minimize the stress that may cause due to the captive nature.

Experimental animals

Six adult tortoises and turtles from each, clinically healthy and in good condition, were kept at the Tortoise holding facility, Department of Livestock and Avian Sciences, Wayamba University of Sri Lanka. All the handling and sample procedures were consistent with standard vertebrate protocols and veterinary practices.

Blood sampling

Turtles and tortoises were restrained manually without sedation. Blood samples were collected according to the standard protocols. A total of 1 ml of blood was collected with the use of 23 G × 1 ¼ " hypodermic needle fixed into a 3CC syringe from subcarapacial sinus of black turtle, parker's black turtle, and star tortoise and from the soft area located in between vertebral and marginals of the caudal part of the carapace of flap shelled turtles. As the bodyweights of all the species that were utilized for the study more than 750 gm, obtaining 1 ml of blood for hematological assessments did not exert any negative impact on all the chelonians utilized in the current study.



Figure 1. (a) i and ii carapace and plastron of *M. trijuga thersites*, (b) i and ii carapace and plastron of *M. trijuga parkeri*, (c) i and ii carapace and plastron of *L. punctata punctata*, and (d) i and ii carapace and plastron of *G. elegans*.

Blood cell morphology

Three blood smears were prepared from each individual and air dried. Smears were stained using Leishman–Gram staining protocol and observed under the ordinary light microscope (Magnification power: $\times 100$ for differential WBC counts and WBC, RBC cell length, width, and nuclear length, and width of each species.

Estimation of cellular dimensions

Numerical data were presented as the mean \pm standard deviation (SD). Cell sizes were calculated using following formulae describe by Tosunoglu et al. [16]; RBC cell sizes $EL \cdot EW \pi / 4$, RBC nuclear sizes $NL \cdot NW \pi / 4$.

Results and Discussion

The body weights of freshwater turtles and tortoises ranged from 0.75 to 4.518 kg (1.79 ± 0.75 kg), straight-line carapace length ranged from 19.1 to 39.3 cm (25.94 ± 4.77 cm), and the length of plastron length from 17 to 23.1 cm (19.70 ± 1.82 cm) with no abnormalities on the plastron and carapace were used to perform hematology analyses. The erythrocytes were nucleated and nuclei were located centrally similar to other reptile species. The nuclei of mature erythrocytes were chromophilic. The cytoplasm of mature erythrocytes were appeared light pink in color, homogeneous, and the nuclei were located centrally and stained dark purple under Leishman–Gram stain. Both cells and their nuclei were irregular in *M. trijuga parkeri*. *Melanochelys trijuga thermalis* had oval-shaped red blood cells and their nuclei were irregular in shape. Red blood cells were oval and their nuclei were round in *L. punctata*.

punctata. *Geochelone elegans* had oval-shaped red blood cells and their nuclei were round in shape. *Lissemys punctata punctata* had the highest erythrocyte volume followed by *M. trijuga parkeri*, *M. trijuga thermalis*, and *G. elegans*, respectively (Table 2).

Heterophils were the most abundant leukocyte type among all the species. *Melanochelys trijuga parkeri* had the highest value of heterophils (55%) and the lowest value (48%) was recorded in *G. elegans*. Monocyte, basophil, and eosinophil counts were comparable among testudines (Table 1).

Heterophils of all the species of tortoises: *M. trijuga parkeri*, *M. trijuga thermalis*, *G. elegans*, and *L. punctata punctata* contained large, eosinophilic cytoplasmic granules with a round shape. The cytoplasm was visualized in pink. The nuclei were frequently pushed toward the periphery of the cell and viewed basophilic with dense chromatin. According to the Table 3, *L. punctata punctata* reported with the highest cell width and *M. trijuga parkeri* had the highest cell length.

The basophil was easily identified by its characteristic large and round granules which are deeply stained in purple. These granules are tightly adhered to the centrally located nucleus in all the species. According to the Table 3, *M. trijuga thermalis* and *M. trijuga parkeri* had the highest cell width and length, respectively. The eosinophil contained round eosinophilic, cytoplasmic granules. The nucleus consisted of clumped chromatin and stained purple. The round or oval as well as single or bi-lobed eccentrically placed nucleus within the cytoplasm was found in all the species of Sri Lankan testudines. Table 3 depicts the mean values of sizes of eosinophils and nuclei of four

Table 1. Differential white blood cell counts in peripheral blood in turtles and tortoises kept in captivity.

Blood cells	<i>M. trijuga parkeri</i>			<i>M. trijuga thermalis</i>			<i>L. punctata punctata</i>			<i>G. elegans</i>		
	Ext.	Means	SD	Ext.	Means	SD	Ext.	Means	SD	Ext.	Means	SD
Heterophils	51–59	55.5	3.08	51–53	51.83	1.33	51–55	53.0	1.67	47–50	48.5	1.38
Lymphocytes	26–30	28.5	2.51	27–34	30.5	2.59	25–33	29.67	2.8	34–37	34.5	2.43
Monocytes	11–17	14.83	2.56	14–20	16.17	2.32	14–18	15.33	0.51	12–18	15.67	2.07
Basophils	1–2	0.17	0.41	1–2	1.0	0.89	1–2	1.5	0.55	1–2	0.83	0.98
Eosinophils	1–2	1.0	0.89	0–1	0.5	0.55	0–1	0.5	0.55	0–1	0.5	0.55

Table 2. Erythrocyte dimensions of examined species.

Examined species	EL	EW	L/W	ES	NL	NW	NL/NW	NS	NS/ES
<i>M. trijuga parkeri</i>	19.7 ± 1.25	10.8 ± 0.63	1.83 ± 0.16	167.20 ± 14.80	6.50 ± 0.53	5.3 ± 0.48	1.23 ± 0.13	27.11 ± 3.67	0.16 ± 0.02
<i>M. trijuga thermalis</i>	18.1 ± 0.57	11.0 ± 0.67	1.65 ± 0.08	156.59 ± 12.93	7.70 ± 0.79	4.40 ± 0.52	1.77 ± 0.24	26.68 ± 4.62	0.17 ± 0.03
<i>L. punctata punctata</i>	16.6 ± 0.52	14.2 ± 0.79	1.17 ± 0.06	185.35 ± 13.92	6.98 ± 0.20	5.25 ± 0.35	1.21 ± 0.43	25.78 ± 9.34	0.14 ± 0.05
<i>G. elegans</i>	7.5 ± 0.41	13.25 ± 1.59	0.57 ± 0.07	78.14 ± 10.73	5.34 ± 0.22	7.20 ± 0.35	0.74 ± 0.04	30.26 ± 2.35	0.39 ± 0.07

EL = Erythrocyte Length, EW = Erythrocyte Width, ES = Erythrocyte Size, NL = Nuclear Length, NW = Nuclear Width, NS = Nuclear Size.

Table 3. Differential leukocyte cell and nuclear size in peripheral blood in turtles and tortoises kept in captivity.

	<i>M. trijuga parkeri</i>	<i>M. trijuga thermalis</i>	<i>L. punctata punctata</i>	<i>G. elegans</i>
Heterophils				
Cell width	13.00 ± 2.1	12.33±2.25	15.33±0.82	12.17±0.75
Length	15.00 ± 2.10	12.00±2.37	14.67±1.51	12.33±1.37
Nuclear width	11.83 ± 2.93	7.33±1.37	8.83±0.75	3.17±0.41
Length	5.83 ± 0.98	5.67 ± 2.34	5.67 ± 0.82	6.50 ± 0.55
Basophils				
Cell width	7.83 ± 0.76	8.17 ± 0.29	7.33 ± 0.29	7.33 ± 0.29
Length	10.33 ± 0.58	8.33 ± 1.15	8.67 ± 0.58	8.17 ± 0.29
Nuclear width	4.67 ± 0.29	5.17 ± 0.29	4.00 ± 0.0	5.33 ± 0.58
Length	8.50 ± 0.50	6.00 ± 0.00	5.33 ± 0.29	7.00 ± 0.87
Esonophils				
Cell width	8.00 ± 0.87	7.67 ± 0.58	8.17 ± 0.29	8.17 ± 0.29
Length	8.67 ± 0.58	9.50 ± 1.32	6.67 ± 1.15	9.67 ± 0.58
Nuclear width	5.67 ± 0.29	5.50 ± 0.50	4.17 ± 0.15	6.50 ± 0.87
Length	6.67 ± 1.15	6.33 ± 0.58	5.33 ± 0.29	8.17 ± 0.29
Lymphocytes				
Cell width	9.80 ± 0.45	9.80 ± 0.84	9.67 ± 1.03	9.33 ± 0.52
Length	10.30 ± 1.10	8.80 ± 1.48	9.50 ± 0.84	9.83 ± 0.98
Nuclear width	9.00 ± 0.00	7.50 ± 1.00	8.83 ± 0.75	7.58 ± 0.49
Length	7.80 ± 0.45	7.20 ± 0.45	7.00 ± 0.63	8.00 ± 0.55
Monocytes				
Cell width	13.40 ± 1.14	12.20 ± 0.84	13.20 ± 1.30	15.40 ± 3.13
Length	12.60 ± 3.58	12.40 ± 0.89	12.20 ± 1.30	13.20 ± 1.48
Nuclear width	10.20 ± 1.92	9.00 ± 1.87	8.60 ± 0.55	11.60 ± 1.95
Length	8.80 ± 0.45	8.00 ± 2.92	10.00 ± 0.00	9.00 ± 0.71

testudines. *Lissemys punctata punctata* had the lowest eosinophil cell length, nuclear width, and length.

The lymphocytes of testudines consisted of a small amount cytoplasm stained in blue and a round shaped nucleus. Monocyte of *M. trijuga parkeri*, *M. trijuga thermalis*, *G. elegans*, and *L. punctata punctata* consisted of a considerable amount of light blue-gray, finely granular or vacuolated cytoplasm, and a bean-shaped nucleus. *Lissemys punctata punctata* has the highest nuclear length and lowest nuclear width (Table 3). The thrombocytes showed an ellipsoidal shape and consisted of a round densely stained nucleus. Thrombocytes appeared as dark purple in color under the gram staining. Thrombocytes appeared as clumps in *M. trijuga parkeri* and *G. elegans*; as single cells in *M. trijuga thermalis* and *L. punctata punctata*.

Hematological information can be used as a quick tool to assess animals' health. The changes in hematologic parameters are reported to be associated with inflammatory and hemoparasitic diseases in chelonians [9]. Furthermore, sex, age, season, geographic sites, reproductive stages, and

sample collection method also affect for blood parameter variations [3,19]. Therefore, the knowledge on hematologic values of testudines is important for managing their populations in the captive environment.

The erythrocytes play a vital role in carrying oxygen and carbon dioxide, thus its size is an indicator that reflects the position of that particular species on the evolutionary scale. The erythrocytes of mammals are smaller and do not contain nuclei when compared with larger erythrocytes with the nucleus in lower vertebrates. Among the fresh water turtles and tortoise species, which were examined in this study, fresh water turtles had larger erythrocytes and nuclei than terrestrial *G. elegans* (Table 2). *Melanochelys trijuga parkeri*, *M. trijuga thermalis*, and *L. punctata punctata* had more ellipsoidal nuclei than terrestrial species in relation to the L/W ratio, though the fresh water species had more ellipsoidal nuclei than *G. elegans* in relation to NL/NW ratio. Thus, it can be concluded that *G. elegans* had more convenient erythrocytes for gas exchange than fresh water turtles. This finding agrees with previous findings by Javanbakht et al. [9].

The lymphocytes have various functions, including responding to foreign invaders by producing antibodies within the reptilian body. Reptilian leukocytes have shown morphological variations within the class which may lead to difficulty in classification [12]. In our study, we identified heterophils with large, round nucleus that was un-lobulated, located eccentrically in the cytoplasm with dense chromatin. It is difficult to distinguish heterophils and eosinophils in the chelonian blood due to their similar appearance [15]. The main difference is the shape of granules. Heterophils contain rod-shaped eosinophilic granules in the cytoplasm whereas round eosinophilic granules are abundant in the cytoplasm of eosinophils [1]. Eosinophils consist of a cytoplasm containing numerous, round eosinophilic granules. The round to oval shaped single nucleus was placed eccentrically within the cytoplasm. Heterophils and eosinophils can be easily identified after staining, because eosinophil granules are stained positively with benzidine peroxidase, whereas heterophil granules are not stained [1].

The basophil was easily recognized by its large, round granules which are deeply stained purple and remained tightly adhered to the centrally located nucleus. Saint Girons [14] revealed that small and large lymphocytes are predominantly found in blood smears of different reptile species. In some reptiles and amphibians species, lymphocytes are reported to be the most abundant cells among white blood cells [2]. The lymphocytes of Sri Lankan testudines contained a small amount of blue staining cytoplasm and a round nucleus. The monocyte contained a large amount of light blue gray, finely granular or vacuolated cytoplasm, and bean or heart-shaped nucleus with a dense chromatin pattern. When considering about the thrombocytes, *M. trijuga thermalis* and *L. punctata punctata* are having single platelets. *Melanochelys trijuga parkeri* and *G. elegans* are having platelets as clumps. Thrombocytes are ellipsoidal with centrally located dark-stained nuclei and their cytoplasm is clear. Lopez et al. [20] found that captive adults' animals of ploughshare tortoises (*Astrochelys yniphora*) showed significant differences in white blood cell counts as compared with wild animals and they further suggested these differences are more related to differences of local environmental or dietary factors.

The results of this current study provide novel information related to reference ranges and cell morphology of red and white blood cells of the *M. trijuga thermalis*, *M. trijuga parkeri*, *L. punctata punctata*, and *G. elegans*. These data would be important for further studies that may evaluate the effects of other factors such as sex, nutritional status, and age on hematological parameters in chelonians.

Conclusion

Knowledge on reference ranges of hematological and physiological parameters are critically important for the

diagnosis of diseases and for the evaluation the health status of turtle and tortoise populations. Thus, the current study for the first time provides valuable information on some hematological parameters and morphological characteristics of different blood cells of clinically healthy turtle and tortoise species reared captivity in Sri Lanka.

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Conflict of interest

The authors would like to declare that there is no conflict of interest related to publication of this paper.

Authors' contribution

This work was carried out in collaboration between all the authors. GAP designed the study and involved in field research trials. HNND was conducted the field research trial, data collection, managed the literature searches, interpreted the data, and drafted the manuscript. LJPAPJ took part in preparing and critical checking of this manuscript. GAP supported the research facilities and funding. All the authors read and approved the final manuscript.

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