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Some Haematological Parameters of Wild Caught Warty Toothed Toad *Oreolalax rugosus* (Liu, 1943) (Anura: Megophryidae)

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Abstract: Haematological parameters can not only assess the health status of animals, but also can reflect animal responses to environments. The haematology of wild caught *Oreolalax rugosus* was examined in the present study. Mean erythrocyte counts was 467916 ± 42015 / mm^3, mean haemoglobin concentration was 6.83 ± 0.22 g/100 mL, and mean leukocyte counts was 1812 ± 322 / mm^3. The leukocyte composition was as follows: lymphocytes, 70.50%; monocytes, 11.25%; basophils, 6.83%; eosinophils, 6.17% and neutrophils, 5.25%. Morphometric analysis of erythrocytes revealed the following dimensions: mean length 20.90 ± 0.17 µm, mean width 12.42 ± 0.16 µm, mean size 203.79 ± 3.6 µm^2 and mean EL/EW ratio 1.69 ± 0.02. Meanwhile, the dimensions of the nucleus were as follows: mean length 7.48 ± 0.22 µm, mean width 4.55 ± 0.13 µm, mean size 26.91 ± 1.47 µm^2 and NL/NW ratio 1.64 ± 0.03. The percentage of neutrophils and neutrophil/lymphocyte (N/L) ratio showed significant sex-related differences. Body size, body mass and age exerted no significant effect on the haematological parameters. The haematological parameters of *O. rugosus* were similar with other anuran and these data can be used to monitor and assess the health of this anuran.

Key words: blood cells; haemoglobin concentration; sexual difference; morphometry

Introduction

Decline in amphibian populations has been observed in all regions worldwide (e.g., Hof et al. 2011, Whittaker et al. 2013, Whitfield et al. 2016). Potential causes for such a decline include habitat destruction, alteration and fragmentation, introduced species, overexploitation, infectious diseases, climate change, increased ultraviolet radiation and chemical contaminants (Whittaker et al. 2013). Haematological parameters can reflect animal responses to external and internal environments (Davis 2008) and may vary with the alteration of physiological, pathological, ecological and environmental conditions (Sarasola et al. 2004, Seaman et al. 2005). Thus, haematological parameters act as important indicators of the health status of animals (Khan & Zafar 2005), as well as bioindicators of the environment (e.g., Cabagna et al. 2005, Zhelev et al. 2013, 2016, Priyadarshani et al. 2015).

Numerous haematological studies have been conducted on anuran species (e.g., Arikan & Çiçek 2014, Wei et al. 2015), but no information on the species of *Oreolalax* is available. A total of 18 species have been recognised in the genus of *Oreolalax*, which is widely distributed in south-western China and the adjacent Vietnam (Frost 2017). *Oreolalax rugosus* (Liu, 1943) is one of the species endemic to China. It inhabits mountain streams between 1800 and 3300 m a.s.l. of southern Sichuan Province and northern Yunnan Province (Fei et al. 2009). Populations declining are expected to continue be-

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cause of habitat loss, resulting from clear-cutting and agricultural expansion. Thus, the species has been categorised as a near-threatened species in the International Union for Conservation of Nature (Fei & Wu 2004). In the present study, we reported on some haematological parameters of wild caught *O. rugosus*. This study aimed to describe the morphological characteristics of different peripheral blood cells and some haematological parameters of the peripheral blood, as well as to provide baseline data for monitoring and assessment of the health status of this anuran. We also evaluated the sexual dimorphism of haematological parameters and the relationships of snout-vent length (SVL) with body mass, age and haematological parameters.

Materials and Methods

Twelve healthy *Oreolalax rugosus* adults (5 males and 7 females) were used in the present study. The specimens were collected from Nixi (27°58′20.02″N, 99°35′19.62″E, 3477 m a.s.l.), Shangri-La County, Yunnan Province, China. All experiments were carried out according to the protocols approved by the Ethics Committee of Animal Experiments at the Henan University of Science and Technology and all animals used in the study were treated humanely and ethically, following the guidelines of the China Council on Animal Care.

Prior to blood collection, the body mass and SVL of each individual were measured. The longest phalange of the left hindlimb of each individual was clipped for age determination, following the method used by Sun et al. (2016). Blood samples were collected from the toads through cardiac puncture. Blood smears were prepared using the push/slide technique, in order to examine the morphology and morphometry of blood cells. Dried blood smears were stained with Wright's stain and observed under a light microscope (Olympus CX31). Different types of blood cells were identified. A total of 100 leukocytes were counted in the blood smear of each individual for differential leukocyte count. The neutrophil-to-lymphocyte (N/L) ratio for each individual was calculated in accordance with the observed proportions. For the morphometric study of erythrocytes on the basis of its size, 100 erythrocytes were taken randomly from each individual and the dimensions were measured using the Biolife Std microscopic image analysis software. The dimensions included the erythrocytes length (EL) and width (EW), as well as the nucleus length (NL) and width (NW). The area of the erythrocytes (EA) was calculated using the formula $EA = ELEW\pi/4$ and the area of the nucleus (NA) was determined by the formula $NA = NLNW\pi/4$ (Tosunoğlu et al. 2011). The shape of cells and nuclei were compared using the EL/EW and NL/NW ratios (Tok et al. 2009). Erythrocyte count was determined with Neubauer haemocytometers, viewed under an Olympus CX31 light microscope. Leukocyte count was calculated using the proportion of erythrocytes to leukocyte in ten randomly selected fields with the aid of a grid ocular micrometer. Haemoglobin (Hb) concentration was determined using Sahil's haemometer. Cell photomicrographs were taken with a mounted Olympus DP26 digital camera.

Prior to statistical analysis, all variables were tested for normality and homogeneity (eosinophil needed to be cosine-transformed). One-way ANOVA was used to compare the sexual differences in haematological parameters. Linear regression was performed to evaluate the relationships of haematological parameters with SVL, body mass and age. The significance level was set to $\alpha = 0.05$. Statistical analyses were performed using SPSS (SPSS Inc., Chicago, Illinois, USA, Version 22.0).

Results

The blood cells of *Oreolalax rugosus* are shown in Fig. 1. Erythrocytes were large ellipsoidal cells, with an ellipsoidal nucleus located at the centre or an eccentric nucleus. The cytoplasm was stained light yellowish pink and the nucleus was stained dark purplish blue with Wright's stain (Fig. 1A). The following measurements of the cells were obtained: mean length 20.07-22.11 µm, mean width 11.8-3.37 µm, mean area 181.41-221.61 µm^2 and mean EL/EW ratio 1.57-1.80. In addition, the following measurements of the nuclei were determined: mean length, 6.43-9.02 µm, mean width, 3.92-5.14 µm, mean area, 19.80-35.67 µm^2 and mean NL/NW ratio 1.43-1.79. The erythrocyte morphometric data of females and males are shown in Table 1. Although variations in morphometric data were observed between sexes, these differences not statistically significant ($p > 0.05$).

According to their morphological characteristics, leukocytes were divided into lymphocytes, monocytes, eosinophils, basophils and neutrophils. The lymphocytes were round or spherical, containing small amounts of cytoplasm; the cytoplasm was stained dark purple, and the nucleus was stained dark purplish blue. The lymphocytes had two different sizes (Figs. 1B, 1C). The monocytes had a kidney- or horseshoe-shaped nucleus and the kidney-shaped nuclei were at least half as big as the cell. The cytoplasm was stained reddish-brown, whereas the nuclei stained dark purplish-blue (Fig. 1D). The eosinophils were also spherical, with some reddish and shiny granules in their cytoplasm (Figure 1E). The basophils were small and spherical and characterised by

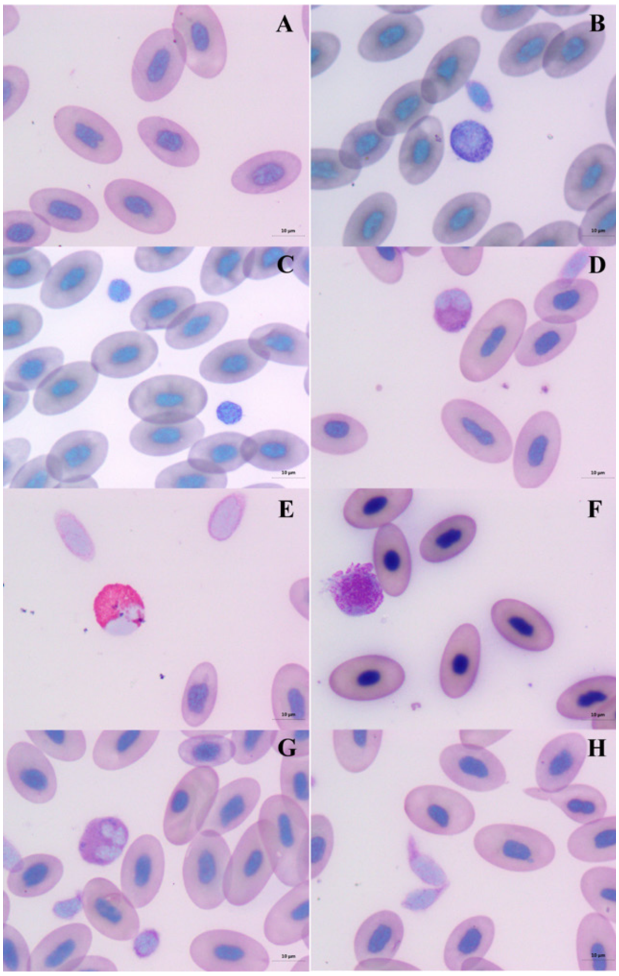

Fig. 1. Blood cells of *Oreolalax rugosus*. A. erythrocyte; B. large lymphocyte; C. small lymphocyte; D. monocyte; E. eosinophil; F. basophil; G. neutrophil; H. thrombocyte.

the presence of basophilic (dark purple) granules in the cytoplasm (Fig. 1F). Neutrophils were spherical, with a multiple-lobed or segmented nucleus. The cytoplasm was stained reddish-brown, whereas the nucleus was stained dark purplish-blue (Fig. 1G).

The thrombocytes were long and fusiform, with a large and ovoid nucleus. Clear cytoplasm was rarely observed and stained light yellowish-pink, whereas the nuclei stained a purplish-blue colour (Fig. 1H).

The erythrocyte count, leukocyte count, Hb concentration and the percentages of each types of leukocytes in both sexes are presented in Table 2. Erythrocyte count and Hb concentration were higher in females than in males, as opposed to leukocyte count. These differences were not statistically significant ($p > 0.05$). The percentages of neutrophil, basophil, monocyte and N/L ratio were larger in females than in males (Table 2); however, only the percentage of neutrophils and N/L ratio exhibited marked sexual difference ($F_{1,10} = 6.074$, $p = 0.033$, $F_{1,10} = 6.183$, $p = 0.032$, respectively).

Table 1. Morphometric data of erythrocytes in *Oreolalax rugosus*.

Charac-ters	Sex	Mean	SD	Range
Erythrocyte length (EL, μm)	Female	20.80	0.26	20.07-21.65
	Male	20.96	0.23	20.13-22.11
	Both sexes	20.90	0.17	20.07-22.11
Erythrocyte width (EW, μm)	Female	12.43	0.23	11.70-13.03
	Male	12.41	0.23	11.48-13.37
	Both sexes	12.42	0.16	11.48-13.37
Erythrocyte area (EA, μm^2)	Female	207.86	4.01	199.17-220.51
	Male	200.88	5.45	181.41-221.61
	Both sexes	203.79	3.6	181.41-221.61
EL/EW	Female	1.69	0.04	1.57-1.80
	Male	1.69	0.02	1.59-1.75
	Both sexes	1.69	0.02	1.57-1.80
Nucleus length (NL, μm)	Female	7.50	0.25	6.76-8.31
	Male	7.45	0.35	6.43-9.02
	Both sexes	7.47	0.22	6.43-9.02
Nucleus width (NW, μm)	Female	4.56	0.21	4.01-5.14
	Male	4.55	0.18	3.92-5.11
	Both sexes	4.56	0.13	3.92-5.14
Nucleus area (NA, μm^2)	Female	26.92	1.67	22.18-32.21
	Male	26.91	2.34	19.80-35.67
	Both sexes	26.91	1.47	19.80-35.67
NL/NW	Female	1.65	0.05	1.47-1.74
	Male	1.64	0.04	1.43-1.79
	Both sexes	1.64	0.03	1.43-1.79

Females had larger values for SVL, body mass and age than males (Table 3). One-way ANOVA suggested that only SVL exhibited sexual dimorphism ($F_{1,10} = 5.479$, $p = 0.041$). Regression analyses indicated that there were no significant differences between haematological parameters and SVL, body mass, as well as age (Table 4).

Discussion

The composition and morphology of *O. rugosus* blood cells were consistent with those of other amphibian species. The peripheral blood cells of amphibians consist of erythrocytes, leukocytes and thrombocytes. Leukocytes can also be subdivided in lymphocytes, monocytes, eosinophils, basophils and neutrophils (Allender & Fry 2008, Arikan & Çiçek 2014, Meesawat et al. 2016). The blood cells in amphibians exhibit a similar morphology in terms of their cell shape, nucleus shape and granules in the cytoplasm (Meesawat et al. 2016). Generally, anuran erythrocytes exhibit an ellipsoidal shape (Szarski & Czopek 1966). The morphology of each type of leukocyte has been conserved across vertebrate taxa, except for that of neutrophils, which have been replaced with heterophils in birds and reptiles (Davis et al. 2008). Arikan

Table 2. Haematological parameters of *Oreolalax rugosus*.

Characters	Sex	Mean	SD	Range
erythrocyte (mm^3)	Female	501000	76638.76	315000-695000
	Male	444285	50278.138	310000-655000
	Both sexes	467916	42015	310000-695000
Hb (g/100ml)	Female	7.04	0.51	5.5-8.5
	Male	6.67	1.55	6.2-7.25
	Both sexes	6.83	0.22	5.5-8.5
Leucocyte (mm^3)	Female	1374	319.24	642-2565
	Male	2125	488.41	518-3936
	Both sexes	1812	322	518-3936
Neutrophil (N, %)	Female	7.20	0.58	6-9
	Male	3.86	1.06	1-9
	Both sexes	5.25	0.81	1-9
Lymphocyte (L, %)	Female	66.00	3.65	56-75
	Male	73.71	3.25	60-83
	Both sexes	70.50	2.59	56-83
Basophil (B, %)	Female	7.8	2.40	3-17
	Male	6.14	1.44	1-12
	Both sexes	6.83	1.26	1-17
Eosinophil (E, %)	Female	5.20	1.53	2-11
	Male	6.86	1.90	4-18
	Both sexes	6.17	1.25	2-18
Monocyte (M, %)	Female	13.8	2.35	6-19
	Male	9.43	1.89	4-17
	Both sexes	11.25	1.55	4-19
N/L	Female	0.11	0.01	0.09-0.13
	Male	0.06	0.02	0.02-0.15
	Both sexes	0.08	0.01	0.01-0.15

Table 3. Snout-vent length (SVL), body mass and age of *Oreolalax rugosus*.

Characters	Females (ranges)	Males (ranges)
Snout-vent length (mm)	52.39±3.27 (41.08-59.26)	45.20±1.20 (41.39-48.61)
Body mass (g)	13.31±2.59 (4.95-18.86)	8.64±1.10 (5.55-12.64)
Age (year)	4.20±0.58 (3-6)	3.43±0.30 (3-5)

Table 4. Regression results of haematological parameters and SVL, body mass and age in *Oreolalax rugosus*.

Characters	SVL	Mass	Age
erythrocyte (mm^3)	$F_{1,10}$=0.291, p=0.161	$F_{1,10}$=2.912, p=0.119	$F_{1,10}$=0.515, p=0.490
Hb (g/100ml)	$F_{1,10}$=3.777, p=0.081	$F_{1,10}$=3.881, p=0.077	$F_{1,10}$=0.321, p=0.584
leucocyte (mm^3)	$F_{1,10}$=0.096, p=0.763	$F_{1,10}$=0.020, p=0.891	$F_{1,10}$=0.375, p=0.554
Neutrophil (%)	$F_{1,10}$=0.004, p=0.953	$F_{1,10}$=0.164, p=0.694	$F_{1,10}$=0.048, p=0.831
Lymphocyte (%)	$F_{1,10}$=2.447, p=0.149	$F_{1,10}$=2.788, p=0.126	$F_{1,10}$=0.103, p=0.755
Basophil (%)	$F_{1,10}$=0.383, p=0.550	$F_{1,10}$=0.731, p=0.412	$F_{1,10}$=0.119, p=0.737
Eosinophil (%)	$F_{1,10}$=0.106, p=0.752	$F_{1,10}$=0.145, p=0.711	$F_{1,10}$=0.122, p=0.735
Monocyte (%)	$F_{1,10}$=3.551, p=0.089	$F_{1,10}$=4.060, p=0.072	$F_{1,10}$=0.438, p=0.523
N/L	$F_{1,10}$=0.045, p=0.837	$F_{1,10}$=0.004, p=0.949	$F_{1,10}$=0.026, p=0.875
Erythrocyte length (EL, μm)	$F_{1,10}$=0.235, p=0.638	$F_{1,10}$=0.369, p=0.557	$F_{1,10}$=0.228, p=0.643
Erythrocyte width (EW, μm)	$F_{1,10}$=0.348, p=0.568	$F_{1,10}$=0.488, p=0.501	$F_{1,10}$=0.175, p=0.685
Erythrocyte size (ES, μm^2)	$F_{1,10}$=0.011, p=0.917	$F_{1,10}$=0.007, p=0.936	$F_{1,10}$=0.006, p=0.938
EL/EW	$F_{1,10}$=0.245, p=0.632	$F_{1,10}$=0.021, p=0.888	$F_{1,10}$=0.265, p=0.618
Nucleus length (NL, μm)	$F_{1,10}$=0.057, p=0.817	$F_{1,10}$=0.111, p=0.746	$F_{1,10}$=0.042, p=0.842
Nucleus width (NW, μm)	$F_{1,10}$=0.213, p=0.654	$F_{1,10}$=0.034, p=0.858	$F_{1,10}$=0.223, p=0.647
Nucleus size (NS, μm^2)	$F_{1,10}$=0.070, p=0.797	$F_{1,10}$=0.088, p=0.773	$F_{1,10}$=0.053, p=0.822
NL/NW	$F_{1,10}$=0.062, p=0.808	$F_{1,10}$=0.051, p=0.825	$F_{1,10}$=0.641, p=0.442

& Çıçek (2014) indicated that thrombocytes can be confused with small lymphocytes. However, the two can be easily distinguished: thrombocytes generally form clusters in blood smears, have an elongated oval to fusiform shape and fairly abundant and almost colourless cytoplasm; lymphocytes are usually round and have less abundant and more basophilic cytoplasm (Allender & Fry 2008). Thrombocytes often form clumps in blood smears, indicating their function in coagulation (Rouf 1969).

The morphological data of erythrocytes and the haematological parameters of *O. rugosus* fell within the ranges reported for other anuran species. However, the percentages of the five types of leukocytes markedly differed from those of other anuran species. Although the percentage of each type of leukocytes vary among species, generally lymphocytes are the most abundant leukocytes, followed by neutrophils, basophils, eosinophils and monocytes (Davis 2009). In the present study, the position of monocytes and neutrophils were exchanged. Differential leucocyte count provides information about the immunological status of an individual (Zhelev et al. 2013, Das & Mahapatra 2014). Monocytes are long-lived phagocytic cells associated with defence against infection and bacteria (Davis et al. 2008). The registered increase in the percentage of monocytes suggested that *O. rugosus* has a high level of defence against infections and bacteria.

The percentage of neutrophils and the N/L ratio indicated sexual difference (females with larger values than males) in the present study. Sexual difference, a production of evolution by natural selection, commonly occurs in the animal kingdom (Cox et al. 2007, Kupfer 2007). Sex-related differences in haematological parameters have been reported for some anurans. Males *Pseudepidalea viridis* have significantly higher haemoglobin, haematocrit and mean cell volume when compared with females (Gül et al. 2011). Likewise, male *Polypedates maculatus* have significantly larger erythrocyte breadth, eosinophil and neutrophil size and area, mean corpuscular volume (MCV), mean corpuscular Hb concentration (MCHC), monocyte and eosinophil counts as compared to female; however, females have significantly higher Hb concentration as well as percentages of basophils and neutrophils (Mahapatra et al. 2012). Erythrocyte count, length and erythrocyte breadth; percentage of neutrophils; N/L ratio; area of basophils; diameters of large lymphocytes and eosinophils have showed significant differences between sexes in *Polypedates teraiensis* (Das & Mahapatra 2014). Male *Hoplobatrachus rugulosus* have a significantly higher packed cell volume (PCV), more lymphocytes and more neutrophils than in females (Meesawat et al. 2016). However, no sexual difference in haematological parameters have been found in other species, such as *Pelodytes caucasicus* (Arikan et al. 2003), *Rana macrocnemis* (Arserim & Mermer 2008), *Acris c. crepitans* (Davis & Durso 2009), *R. ridibunda*, *R. dalmatina*, *Bufo bufo*, *Hyla arborea* and *Pelobates syriacus* (Gül & Tok 2009), *Bombina bombina* (Arikan et al. 2010). These results indicate that sexual dimorphism in the haematological parameters varies among species and haematological variables. Neutrophils are the primary phagocytic leukocyte. Stress can increase the number of neutrophils and decrease the number of lymphocytes. Thus, the N/L ratio has long been used to reflect levels of physiological stress in all vertebrates (Davis et al. 2008) and more specificly in anuran species (Zhelev et al. 2013). Whether the sexual difference in these two parameters contribute to the difference of physiological stress between sexes, needs to be confirmed in the future.

Haematological parameters have been found to exhibit no significant correlation with SVL, body mass and age in the present study. These results were consistent with those obtained in other studies (e.g., Davis & Durso 2009, Arikan & Çıçek 2014). However, SVL, body mass and age have been known to affect haematological parameters in some anurans. Significant differences in both cell and nuclear size have been found between juvenile and adult *Odontophrynus americanus* (see Grenat et al. 2010). The erythrocyte morphology and leukocyte profile of *Polypedates maculatus* vary from larval development to metamorphosis (Das & Mahapatra 2015). Previous studies have found significant correlations between SVL and Hb%, PCV, MCV and leukocyte count; body mass and Hb%, PCV, MCV, leukocyte count and erythrocyte count in *P. maculatus* (see Mahapatra et al. 2012).

To conclude, this study is the first to report on the haematological parameters in *O. rugosus*, providing baseline data for wild *O. rugosus*, which can be used to assess and monitor the health of this anuran. Moreover, the results revealed that the percentage of neutrophils and N/L ratio of females were significantly higher than those of males. However, the haematological parameters exhibited no significant correlation with SVL, body mass and age.

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