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Blood Cell Morphology and Leukocyte Profile of the Himalayan Newt *Tylototriton verrucosus* Anderson, 1871 (Urodela: Salamandridae) in Thailand

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ABSTRACT.– We examined the blood cell morphology and leukocyte profile in eight adult individuals (three female and five male newts) of the Himalayan newt (*Tylototriton verrucosus*) found at Doi Chang, Chiang Rai Province, Thailand. The hematology of *T. verrucosus* was found to be generally similar to other urodeles, despite small differences being observed in the cellular shape and size. The peripheral blood cells of *T. verrucosus* were erythrocytes, leukocytes (lymphocytes, monocytes, neutrophils, eosinophils, and basophils), and thrombocytes. The mean erythrocyte width and length was 20.18 ± 2.58 and 33.15 ± 3.91 µm, respectively. The mean diameter of the lymphocytes, monocytes, neutrophils, eosinophils, and a single basophil was 20.38 ± 3.84, 27.73 ± 4.44, 21.87 ± 2.79, 23.86 ± 3.75, and 28.62 µm, respectively. From the differential leukocyte count, the percentage of these leukocytes was 47.94, 0.44, 46.31, 5.25, and 0.06%, respectively. The mean thrombocyte width and length was 11.56 ± 1.67 and 22.62 ± 3.36 µm, respectively. This study will be used as baseline data for other hematological studies of the newt genus *Tylototriton* in the future.

KEY WORDS: baseline data, hematology, peripheral blood cells, South East Asia, tailed amphibian

INTRODUCTION

The newt genus *Tylototriton* Anderson, 1871 is classified as a vertebrate in the phylum Chordata; class Amphibia; order Urodela; family Salamandridae. They range across eastern Himalayas to central and southern China, and southwards through southeastern Asia (Hernandez and Pomchote, 2020c; Pomchote et al., 2020b). At present, five species have been reported in Thailand: *Tylototriton uyenoi* and *T. panhai* Nishikawa, Khonsue, Pomchote, and Matsui, 2013; *T. anguliceps* Le, Nguyen, Nishikawa, Nguyen, Pham, Matsui, Bernardes, and Nguyen, 2015; *T. verrucosus* Anderson, 1871; and *T. phukhaensis* Pomchote, Khonsue, Thammachoti, Hernandez, Suwannapoom, and Nishikawa, 2020. They are distributed along the high mountain ranges in the northern (*T. uyenoi*, *T. anguliceps*, *T. verrucosus*, and *T. phukhaensis*), north-eastern (*T. panhai*), and western (*T. uyenoi*) regions of Thailand (Pomchote et al., 2008; Nishikawa et al., 2013; Hernandez, 2016; Hernandez et al., 2019; Hernandez and Pomchote, 2020a, 2020b, 2020c; Pomchote et al., 2020a, 2020b). However, only *T. verrucosus* is in the amphibian wildlife protection list of Thailand (Department of National Parks, Wildlife and Plant Conservation, 2021).

In Thailand, *Tylototriton* species reside in the high mountainous areas at an altitude of more than 1000 m above mean sea level (amsl), where they are found in national parks or wildlife sanctuaries. However, only a few individuals of *T. verrucosus* have been found at Doi (= Mountain in Thai language) Chang, Chiang Rai Province, which is located outside the government protected enclosures. Thus, this population may be at risk of decline due to human activities (Pomchote et al., 2020a). To assess the health status of amphibians, hematological investigations (especially blood cell morphology) have been considered as a biomarker for environmental stress (Davis et al., 2009; Salinas et al., 2017; Zhelev et al., 2016, 2017, 2021). However, there are no detailed data on the hematology of either *T. verrucosus* or other *Tylototriton* species.

The aim of this study was to examine the blood cell morphology and leukocyte profile in *T. verrucosus*. The data obtained from this study is the first report for *Tylototriton* species and so represents the baseline data for, once expanded to other collection areas and dates, monitoring the health status of *Tylototriton* species for future conservation plans.

MATERIALS AND METHODS

Eight adult specimens of *T. verrucosus* (three female and five male newts) were collected from the breeding pond at Doi Chang, Chiang Rai Province, northern Thailand (Fig. 1) at an altitude of 1500 m amsl from 29–30 July 2018, which was during the breeding season of Thai *Tylototriton* species (Pomchote et al., 2008). The breeding pond was an artificial pond that was used to support agricultural activities, such as crop cultivation. Surrounding areas were hills covered in evergreen forest (details of the breeding pond are in Figure 5 of Pomchote et al., 2020a). The specimens were sexed based on their sexual cloacal characters (Pomchote

FIGURE 1. *Tylototriton verrucosus* from Doi Chang, Chiang Rai Province, Thailand. Photo by Porrawee Pomchote

et al., 2008). Following previous studies (Pomchote et al., 2020a), the samples were anesthetized by immersion in a solution of 5 g/L tricaine methane sulfonate (MS-222) for about five minutes. Blood samples were collected by removing the tail tips or tail amputation (Campbell, 2015; Forzán et al., 2017) and waiting for a drop of blood to fall onto the glass slides. After the blood had dropped onto the glass slides, it was smeared as a thin layer, air dried, fixed in absolute methanol for 1 min, and then stained with Giemsa’s staining solution as reported (Bain and Lewis, 2012), and mounted with Permount. The blood cell morphology and leukocyte profile were subsequently studied using light microscopy with a 40x objective lens in the laboratory (Salakij, 2005). After completing the blood collection, and when fully conscious, all the newts were released on land near their breeding pond.

The blood cell morphology and morphometry were examined using the Image-Pro Plus (ver. 6.00 software). The morphometric study was expressed in µm, and based upon examination of areas of the blood smear without overlapping blood cells. The following measurement parameters were modified from Meesawat et al. (2016). For erythrocytes, 100 randomly selected cells from each individual were measured for their width, length, erythrocyte area (EA; width x length x π/4), nuclear width, nuclear length, nuclear area (NA; nuclear width x nuclear length x π/4), and nucleocytoplasmic ratio (NA/EA). For leukocytes, 30 cells from each individual were measured. The diameter of the 30 randomly selected cells for each of lymphocytes, monocytes, neutrophils, and eosinophils, plus the sole example of a basophil, were measured in the same way. From the blood smear slide of each newt, 200 leukocytes were counted for the differential leukocyte count (Salakij, 2005). Thirty thrombocytes were randomly selected from each individual for measurement of their width and length (Kuramoto, 1981).

Because of the small number of animals that were found, data from both sexes were pooled. All morphometric data and differential leukocyte count are shown as the mean ± one standard deviation (SD) and range, as analyzed using the SPSS for Window version 22 software.

RESULTS

The morphological characteristics of the peripheral blood cells (PBCs) showed that three cell types were identified (erythrocytes, leukocytes, and thrombocytes). The erythrocytes were elliptical in shape with a light blue or colorless stained cytoplasm. The nucleus was generally elliptical with dense basophilic chromatin, and was located in the center of the cell (Fig. 2A). The mean width and length of the erythrocytes was 20.18 ± 2.58 and 33.15 ± 3.91 µm, respectively. The mean width and length of the nuclei was 9.37 ± 1.36 and 14.45 ± 1.55 µm, respectively, giving a mean nucleocytoplasmic ratio of 0.21 ± 0.04 µm (Table 1).

The leukocytes were divided into agranulocytes and granulocytes. The mean diameters of all types of leukocytes are shown in Table 2. Agranulocytes were classified into lymphocytes and monocytes. The lymphocytes were round in shape and contained a small amount of cytoplasm that was stained light blue or colorless. The nucleus was large, round in shape, stained dark purple, and almost completely filled the cell (Fig. 2B). The cell diameter was 20.38 ± 3.84 µm.

The monocytes were round in shape with a light purple- or blue-grey-stained cytoplasm. The nucleus was eccentric oval or kidney-shaped and located at the rim of the cell (Fig. 2C). The mean cell diameter was 27.73 ± 4.44 µm.

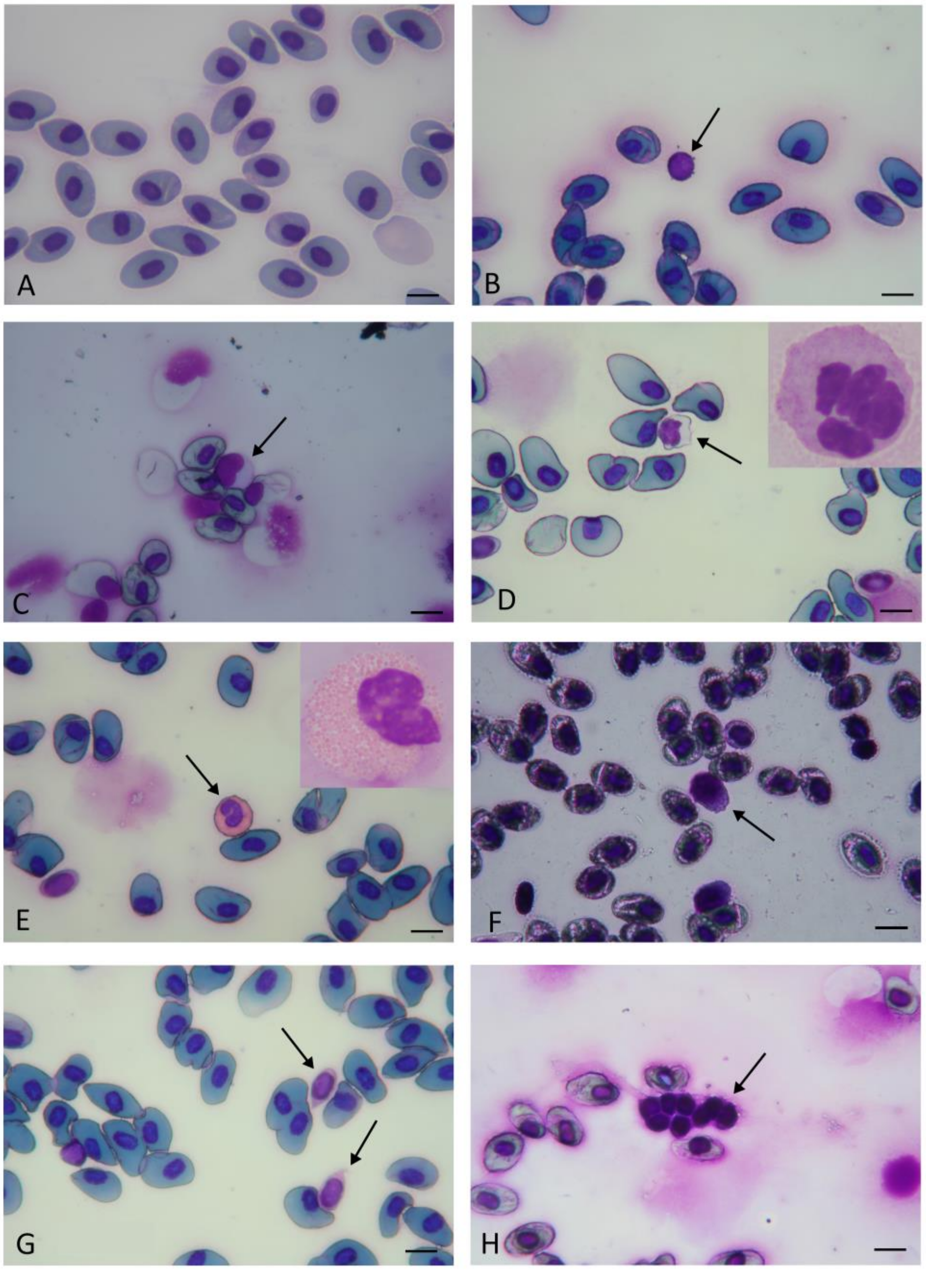

FIGURE 2. The PBCs of *T. verrucosus*. (A) erythrocytes, (B) lymphocyte (arrow), (C) monocyte (arrow), (D) neutrophil (arrow) and its granules, (E) eosinophil (arrow) and its granules, (F) basophil (arrow), (G) thrombocytes (arrows), and (H) group of thrombocytes (arrow). Scale bar = 20 µm; Giemsa stain. Photos by Kotchanun Bunjerdluk

Granulocytes were subdivided into neutrophils, eosinophils, and basophils. The neutrophils were round in shape, with an uncertain-shaped and multiple-lobed nucleus

TABLE 1. Morphometric data of the erythrocytes and thrombocytes of *T. verrucosus* from Doi Chang, Chiang Rai Province, Thailand

Type of blood cell	Character	n	Mean	SD	Range
Erythrocyte	Width (µm)	8	20.18	2.58	13.15–30.58
	Length (µm)	8	33.15	3.91	24.45–50.41
	Erythrocyte area (EA; µm^2)	8	528.84	112.59	282.30–924.34
	Nuclear width (µm)	8	9.37	1.36	5.52–13.79
	Nuclear length (µm)	8	14.45	1.55	10.52–21.54
	Nuclear area (NA; µm^2)	8	107.33	24.44	55.58–218.17
	Nucleocytoplasmic ratio (NA/EA)	8	0.21	0.04	0.11–0.33
Thrombocyte	Width (µm)	8	11.56	1.67	8.06–20.92
	Length (µm)	8	22.62	3.36	14.21–30.71

that was dark-blue stained and not located at the center of the cell. The cytoplasm contained fine granules and stained light purple (Fig. 2D). The mean cell diameter was 21.87 ± 2.79 µm. The eosinophils were round in shape and their cytoplasm had densely spherical and orange-red-stained granules of various sizes. The nucleus had 1 or 2 lobes, was stained dark-purple, and located at the periphery of the cell (Fig. 2E). The mean cell diameter was 23.86 ± 3.75 µm. Only one basophil was recorded, and this was elliptical in shape, with a round-shaped nucleus, and a dark purple-stained cytoplasm containing large granules (Fig. 2F). The cell diameter was 28.62 µm.

The differential leukocyte count revealed that lymphocytes were the most abundant leukocytes (47.94%), followed by neutrophils (46.31%), eosinophils (5.25%), monocytes (0.44%), and basophils (0.06%), respectively, (Table 3).

The thrombocytes were elliptical to spindle-like in shape, with a large and elliptical nucleus. The cytoplasm was stained blue or light purple (Fig. 2G). Sometimes the cells clumped together in blood smears (Fig. 2H). The mean cell width and length was 11.56 ± 1.67 and 22.62 ± 3.36 µm, respectively, (Table 1).

DISCUSSION

The present study is the first report of the PBCs based on their morphology and differential leukocyte count in the urodelan newt genus *Tylototriton*. The genus *Tylototriton* is a priority in terms of conservation as most of the species are threatened by extinction (Nishikawa et al., 2013; Hernandez, 2016; Hernandez and Pomchote, 2020a; Pomchote et al., 2020a; AmphibiaWeb, 2021; IUCN, 2021). Thus, the baseline data of their hematology are crucial for monitoring the health status of *Tylototriton* species for future conservation plans.

The morphology of erythrocytes of *T. verrucosus* was rather similar to those of urodeles in terms of shape (Pfeiffer et al., 1990; Tosunoğlu et al., 2008; Arikan and Çiçek, 2010; Tosunoğlu et al., 2011) and size (Arikan and Çiçek, 2010), although some

TABLE 2. Cell diameters of leukocytes of *T. verrucosus* from Doi Chang, Chiang Rai Province, Thailand

Diameter	n	Mean	SD	Range
Lymphocyte (µm)	8	20.38	3.84	13.64–32.17
Monocyte (µm)	8	27.73	4.44	21.39–35.94
Neutrophil (µm)	8	21.87	2.79	16.06–29.88
Eosinophil (µm)	8	23.86	3.75	16.30–33.08
Basophil* (µm)	8	28.62	-	28.62

* Data are derived from a single cell.

differences have been reported, especially in their size. The nuclei of the erythrocytes were located at the center of the cells (Tosunoğlu et al., 2008; Tok et al., 2009; Arikan and Çiçek, 2010; Tosunoğlu et al., 2011; this study). Urodeles have been reported to have larger erythrocyte cells and nuclei than those of anurans (Atatür et al., 1999; Arikan and Çiçek, 2010; Wei et al., 2015), consistent with this study. From previous studies in the three orders of amphibians, the dimensions of erythrocytes varied in size among species (Wei et al., 2015). These variations can be affected by several factors, such as species, sex, habitat, behavior, and environmental stress (e.g., Winthrobe, 1933; Ruiz et al., 1989; Arikan and Çiçek, 2010, 2014; Wei et al., 2015; Salinas et al., 2017; Zhelev et al., 2016, 2017, 2021).

Ruiz et al. (1989) compared some parameters of the erythrocytes between *Bufo spinulosus* (Wiegmann, 1834) (now named *Rhinella spinulosa*) that resided in low (from near sea level up to 2700 m amsl) and high (3200 up to close to 4500 m amsl) altitudes. They found that the highland population had a smaller average erythrocyte size than the lowland population. The smaller-sized erythrocytes may be related to giving a larger surface area to volume ratio to increase the efficiency of gas exchange.

Adult *T. verrucosus* are considered as terrestrial or semi-fossorial. They spend most of the year sheltering under logs, rocks, or under leaf litter, whereas during the breeding season they inhabit still or slowly moving waters with organic debris (AmphibiaWeb, 2021). However, we did not examine the differences based on correlation with their body weight and size, as previous studies have (Vernberg, 1955). Comparing the data of Tosunoğlu et al. (2011) and this present study (Table 1) revealed that terrestrial urodeles had a larger average erythrocyte size than aquatic and semi-aquatic ones (details of species and size of erythrocytes are in Table 4 of Tosunoğlu et al., 2011).

Previously, *Pelophylax ridibundus* (Pallas, 1771) living in the anthropogenically polluted areas was reported to have smaller erythrocytes with a more rounded shape to the cell and nucleus compared with those living in the less disrupted areas (Zhelev et al., 2016, 2017). In this study, we found that the erythrocytes of *T. verrucosus* were elliptical in shape, but comparative studies on the various degrees of disrupted areas where *T. verrucosus* live should be future investigated.

The mean nucleocytoplasmic ratio in *T. verrucosus* (Table 1) was in the same range as that reported in seven species of urodeles (0.22–0.34) compared with 12 species of anurans (0.10–0.16) (Arikan and Çiçek, 2010). Thus, urodeles, including *T. verrucosus*, had a narrower cytoplasmic surface area than anurans.

TABLE 3. Differential leukocyte count of *T. verrucosus* from Doi Chang, Chiang Rai Province, Thailand

Parameter	n	Mean	SD	Range
Differential lymphocyte (%)	8	47.94	14.87	25.50–66.50
Differential monocyte (%)	8	0.44	0.51	0–1.00
Differential neutrophil (%)	8	46.31	13.8	32.50–73.00
Differential eosinophil (%)	8	5.25	7.27	1.00–18.00
Differential basophil (%)	8	0.06	0.25	0–0.50

The leukocytes in *T. verrucosus*, and in other urodeles, typically showed a more similar morphology but larger sizes than those of anurans (Arikan and Çiçek, 2010). Among the leukocytes, lymphocytes presented as the dominant cells in amphibians (Arikan and Çiçek, 2014), which is consistent with this study on *T. verrucosus* (Table 3). In general, lymphocytes are the smallest leukocyte in amphibians (Wright, 2001; this study).

Regarding monocytes, those from *T. verrucosus* showed similar kidney-shaped nuclei to those of other amphibians (Tosunoğlu et al., 2008; Tok et al., 2009; Arikan and Çiçek, 2010; Tosunoğlu et al., 2011). However, when compared to seven urodele species from Turkey, the mean cell diameter of the monocytes from *T. verrucosus* was larger than those of the seven species of urodeles, at 27.73 µm vs. 21.00 µm, respectively, (Arikan and Çiçek, 2010). The neutrophils of *T. verrucosus* displayed lobed nuclei, as found in most amphibians, and the granules were smaller than those of eosinophils, which is consistent with previous studies (Wright, 2001; Tosunoğlu et al., 2008; Tok et al., 2009; Tosunoğlu et al., 2011). The mean diameter of neutrophils in *T. verrucosus* was not clearly different from those of other urodeles (Arikan and Çiçek, 2010) at 21.87 vs. 22.78 µm, respectively.

The eosinophils of *T. verrucosus* had spherical granules and less lobed nuclei than those of neutrophils, and resembled those found in most amphibians (Arikan and Çiçek, 2010). However, the mean diameter of the eosinophils from *T. verrucosus* (23.86 µm) was larger than those of urodeles (21.13 µm) (Arikan and Çiçek, 2010).

In this study we found only a single basophil cell. It contained a non-lobed nucleus and heavily stained basophilic granules. Although basophils were the prevalent cell type among the leukocytes in some amphibians (Campbell, 2004, 2015), they were the scarcest type of leukocyte in some urodeles (Tosunoğlu et al., 2008; Tok et al., 2009; this study). The nucleus was not located at the center of the cell in *T. verrucosus*, as reported also in *Ambystoma tigrinum* (Green, 1825) (Campbell, 2015), but was centrally located in most amphibians (Arikan and Çiçek, 2010). The diameter of the basophil in *T. verrucosus* was much larger than in the other urodeles (Arikan and Çiçek, 2010) (28.62 µm vs. 19.41 µm, respectively).

The differential leukocyte count of *T. verrucosus* showed that lymphocytes were the most abundant leukocyte cell type, followed by neutrophils, which is in agreement with previous reports in six other species of urodeles: *Notophthalmus viridescens* (Rafinesque, 1820), *Cryptobranchus alleganiensis* (Sonnini de Manoncourt and Latreille, 1801), *Lissotriton vulgaris* (Linnaeus, 1758), *Plethodon cinereus* (Green, 1818), *Lyciasalamandra fazilae* (Başoğlu and Atatür, 1975 "1974"), and *Ommatotriton ophryticus* (Berthold, 1846) (data from Bennett and Daigle, 1983; Solis et al., 2007; Tosunoğlu et al., 2008; Davis and Durso, 2009; Tok et al., 2009; Tosunoğlu et al., 2011; respectively), whereas the relative abundance of the other types of leukocytes varied, depending on the species. However, variations in the number of leukocytes are caused by several factors, such as species, age, sex, season, ecology, and pathology (Davis et al., 2008; Arikan and Çiçek, 2014).

The morphology of the thrombocytes in *T. verrucosus* was similar to those reported in other amphibians (Tosunoğlu et al., 2008; Claver and Quaglia, 2009; Tok et al., 2009; Arikan and Çiçek, 2010; Tosunoğlu et al., 2011; Thrall et al., 2012; Campbell, 2015; Meesawat et al., 2016), being elliptical- to spindle-shaped with a large nucleus. A group of thrombocytes were also observed, as previously mentioned. The length-to-width ratio of thrombocytes was reported to be nearly two in urodeles (Arikan and Çiçek, 2010), consistent with this study, but in anurans this ratio is about 1.5 (Arikan and Çiçek, 2010). Thus, the thrombocytes of urodeles are more ellipsoid than in anurans.

Amphibians are poikilothermic animals and their blood parameters are sensitive to pollution from anthropogenic activities, in terms of the morphology of blood cells (Salinas et al., 2017; Zhelev et al., 2016, 2017, 2021). Salinas et al. (2017) found that *Rhinella arenarum* (Hensel, 1867) populations from the most disturbed site showed a smaller size of erythrocytes and most types of leukocytes than those from the less disturbed site. They suggested that changes in the blood cell morphology could be associated with the presence of some pollutants. Extensive studies on the changes in erythrocyte sizes in *P. ridibundus* populations living in various disturbed areas have been conducted (Zhelev et al., 2016, 2017, 2021). Not only were some of erythrocyte-metric parameters correlated to anthropogenic activities, but also to seasonal changes (Zhelev et al., 2016), and, to a lesser extent, on the type of water basin (static and flowing) (Zhelev et al., 2017). Thus, the authors proposed that hematological investigations of amphibians, especially the blood cell morphology, could be used as reliable biomarkers in environmental assessment. Thus, future research is required to investigate the complex and synergistic correlations between the effects from human activities, such as toxic substances (pesticide, herbicide, insecticide, heavy metal, etc.) and environmental factors (pH, temperature, dissolved oxygen, conductivity, total dissolved solid, etc.) *in situ* (see Zhelev et al., 2016, 2017, 2021).

The Doi Chang area of this study has been used for commercial crop cultivation and is also a famous tourist attraction with abundant tourist accommodation and facilities. These human activities may affect the health status of *Tylototriton* living around this area to some extent, but the impact of human activities is not the subject of this current study. We propose that further research is needed with a larger number of specimens to compare between less and more disturbed sites. However, currently, *T. verrucosus* has only been reported from Doi Chang, Chiang Rai Province. In order to perform an in-depth investigation into the relationship between the physicochemical characteristics of the environment and biological functions, as well as seasonal fluctuations between breeding (in water) and non-breeding (on land) seasons, further field surveys are needed.

In conclusion, the hematology of *T. verrucosus* is generally similar to other urodeles, although small differences were observed in terms of the cell shapes and sizes. The PBCs of *T. verrucosus* were erythrocytes, leukocytes (lymphocytes, monocytes, neutrophils, eosinophils, and basophils), and thrombocytes. Lymphocytes were the most abundant, while basophils were the scarcest type of leukocytes. This study provided the first hematological baseline data of *Tylototriton* species. The intrinsic and extrinsic factors that may affect the morphology of blood cells and leukocyte profile between *T. verrucosus* and other amphibians mentioned in the literature require further investigation.

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