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Hematology of the Lycian Salamander, *Lyciasalamandra fazilae*

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Abstract. We examined some hematological parameters and the cytomorphometry of blood cells (erythrocyte, leucocyte and thrombocyte) in 10 (5 ♂♂, 5 ♀♀) adult Lycian salamanders, *Lyciasalamandra fazilae,* from İztuzu - Muğla, Turkey. In 1 mm^3 of blood, we estimated the erythrocyte count as 1.06x10^5 (0.73x10^5-1.40x10^5), leucocyte count as 2.14x10^3 (1.60x10^3 – 2.87x10^3), haemoglobin amount as 5.00 (4.80-5.20) g/dl, hematocrit value as 36.66 (36.00-38.00) %, mean cell volume as 3281.2 (2714.3-3600.0) fl, mean cell haemoglobin as 451.01 (342.86-520.00) pg, and mean cell haemoglobin concentration as 13.65 (12.63-14.44) %. Small differences were observed in cell sizes, although they are concluded to be individual variations.

Key words: *Lyciasalamandra fazilae*, Urodela, blood smears, hematological parameters

Introduction

The genus *Lyciasalamandra* (*L. luschani*, *L. atifi*, *L. antalyana*, *L. billae*, *L. fazilae*, *L. flavimembris* and *L. helverseni*) ranges from Greece to the south and southwest of Turkey as well as some islands including Kastellorizon, Meyisti, Kekova, and Carpathos (Veith & Steinfartz 2004). The Lycian salamander, *Lyciasalamandra fazilae* (Başoğlu & Atatür 1974), is endemic to southwestern Anatolia. Several studies on the taxonomy (Veith et al. 2001, Veith & Steinfartz 2004), osteology (Özeti 1974), serology (Özeti & Atatür 1979), food habits (Çiçek et al. 2007) and ecology (Gautier et al. 2006) of genus *Lyciasalamandra* are available, but there are no detailed data on the hematology of *L. fazilae*.

The majority of the references on the hematology of different urodele species are on blood cell counts and sizes (Szarski & Czopek 1966, Atatür et al. 1998, Arıkan et al. 2003, Tosunoğlu et al. 2008). A previous study (Atatür et al. 1998) only investigated the erythrocyte size of *L. fazilae* together with other urodele species distributed in Turkey.

The purpose of this study was to describe the hematological properties (including cell counts, sizes, haemoglobin contents, hematocrit, mean cell volume, mean cell haemoglobin and mean cell haemoglobin concentration) of the Lycian salamander.

Materials and Methods

The twelve specimens of *Lyciasalamandra fazilae* (7 ♂♂, 5 ♀♀) used in this study were collected from İztuzu - Muğla (36^o 46' N, 28^o 40' E, 105 m a.s.l.), Turkey in March 2006. The live animals had been

anaesthetized with ether. Blood samples of the live specimens were obtained in the laboratory within two days of their capture by means of cardiac ventricular puncture via heparinized hematocrit capillaries (Arıkan et al. 2003).

Erythrocytes (RBC) and leucocytes (WBC) were counted manually with a Neubauer hemocytometer. As diluting solutions, the standard Hayem's solution was used for erythrocytes and aliquots of 1/5000 neutral red solution and 12% formalin in 0.07% physiological saline (Jerrett and Mays 1973) were used for leucocytes,. The latter method is a slight modification of Blain's method (Sturkie 1954). Hematocrit value (HCT) was determined by the micro-hematocrit method. The tubes were then spun in a micro-hematocrit centrifuge for 5 min. at 13,000 rpm and the packet cell volume calculated with a hematocrit reader. Haemoglobin concentration (Hb) was measured with a Sahli hemometer. In this apparatus, 100% corresponds to 14.5 g Hb/100 ml blood (Tanyer 1985). The mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were calculated according to Wintrobe's formula (1933).

In measuring the morphology and size of blood cells, blood smears, which were prepared by Wright's stain, were used. Blood cells were measured using MOB-1-15x micrometric ocular apparatus. Length (L) and width (W) of 40 randomly-chosen erythrocytes as well as nucleus length (NL) and nucleus width (NW) were measured on each blood smear. Erythrocytes (S) and their nucleus sizes (NS) were calculated according to the formula $LW\pi/4$, and $NLNW\pi/4$. Cell and nucleus shapes were compared according to L/W and NL/NW ratios while nucleus/cytoplasm comparison was made according to nucleocytoplasmic ratio (N/C). Moreover, leucocytes and thrombocytes (TL, TW) on blood smears of each individual were measured and their sizes were determined.

Statistical analyses were performed by SPSS (ver. 10.00) statistical package program. Since the distribution of data was not significantly different from the normal distribution (Kolmogorov-Smirnov test, $P>0.05$), averages were compared with Student's t test ($P\leq0.05$). Cell photomicrographs were taken with an Olympus CX31 photomicroscope.

Results

The mean erythrocyte count was $1.06x10^5/mm^3$ ($0.73x10^5$ – $1.40x10^5$) (Table 1). Although small differences were observed between males and females in terms of erythrocyte count, this difference was not statistically significant (t= 0.430, df= 10, P= 0.676). The mean leucocyte count per mm^3

Table 1. Hematological parameters of circulating blood of Lycian Salamander. [RBC: red blood cell count, WBC: White blood cell count, Hb: haemoglobin, HCT: hematocrit, MCV: mean cell volume, MCH: mean cell haemoglobin, MCHC: mean cell haemoglobin concentration, n: sample size, SD: standard deviation].

Parameters	Sex	n	Mean	SD	Range
	♂♂	7	$1.08x10^5$	23251.21	$0.83\ x10^5$ – $1.40x10^5$
RBC (1 mm^3)	♀♀	5	$1.03x10^5$	22353.97	$0.73x10^5$ – $1.36x10^5$
	♂♀	12	$1.06x10^5$	22032.34	$0.73x10^5$ – $1.40x10^5$
	♂♂	4	$1.87x10^3$	182.72	$1.60x10^3$ – $2.00x10^3$
WBC (1 mm^3)	♀♀	4	$2.42x10^3$	314.14	$2.20x10^3$ – $2.87x10^3$
	♂♀	8	$2.14x10^3$	376.43	$1.6x10^3$ – $2.87x10^3$
Hb (g/dl)	♂♀	3	5.00	0.200	4.80 – 5.20
HCT (%)	♂♀	3	36.66	1.150	36.00 – 38.00
MCV (fl)	♀♂	3	3281.2	492.25	2714.3 – 3600.0
MCH (pg)	♂♀	3	451.01	94.840	342.86 – 520.00
MCHC (%)	♂♀	3	13.654	0.928	12.63 – 14.44

of blood was found to be $2.14x10^3$±376.43 (Table 1). Significant sexual differences were found in leucocyte count (t= 3.003, df= 6, P=0.024).

The haemoglobin amount was found to be 5.00±0.200 g/dl. The mean hematocrit value was 36.66±1.150 %. The mean cell volume was estimated to be 3281.2±492.25 fl. The mean cell haemoglobin was 451.01±94.840 pg, and the mean cell haemoglobin concentration was calculated to be 13.65±0.92 % (Table 1).

As in other urodeles, the erythrocytes of *Lyciasalamandra fazilae* were oval shaped, 32.00-45.00 µm by 16.00-25.00 µm in size, with an oval nucleus centrally placed, 14.00-20.00 µm by 8.00-13.00 µm in size (Fig.1a, Table 2).

Lymphocytes were spherically shaped, with both small and large lymphocytes in blood smears (Fig.1b). The large lymphocytes had a mean diameter of 26.01 µm (Table 2) and centrally-placed nuclei. Using Wright's stain, lymphocyte cytoplasm stained pale blue and their nuclei dark purple-blue. The small lymphocytes had a mean diameter of 18.26 µm (Table 2) and their large nuclei almost completely filled the cell, with only a thin rim of cytoplasmic ring visible. Lymphocytes were the most abundant leucocytes in blood smears.

The monocytes were similar in size to large lymphocytes, but were not difficult to distinguish from the latter on account of the shape of their nuclei (Fig.1c). Their mean diameter was 29.83 µm (Table 2), with some granulation in their cytoplasm. The kidney shaped nuclei were at least half as big as the cell. With Wright's stain, the cytoplasm coloured light purple, and the nucleus stained dark blue. Monocytes were the third-most abundant leucocytes after lymphocytes and neutrophils.

The neutrophils were spherical with a mean diameter of 27.04 µm (Fig.1d, Table 2). Their cytoplasm stained light blue, and their nuclei a dark purplish blue with Wright's stain. The cytoplasm of neutrophils contained thin granules. The nuclei demonstrated a multi-lobed or segmented structure. They were the second-most abundant leucocytes, after the lymphocytes.

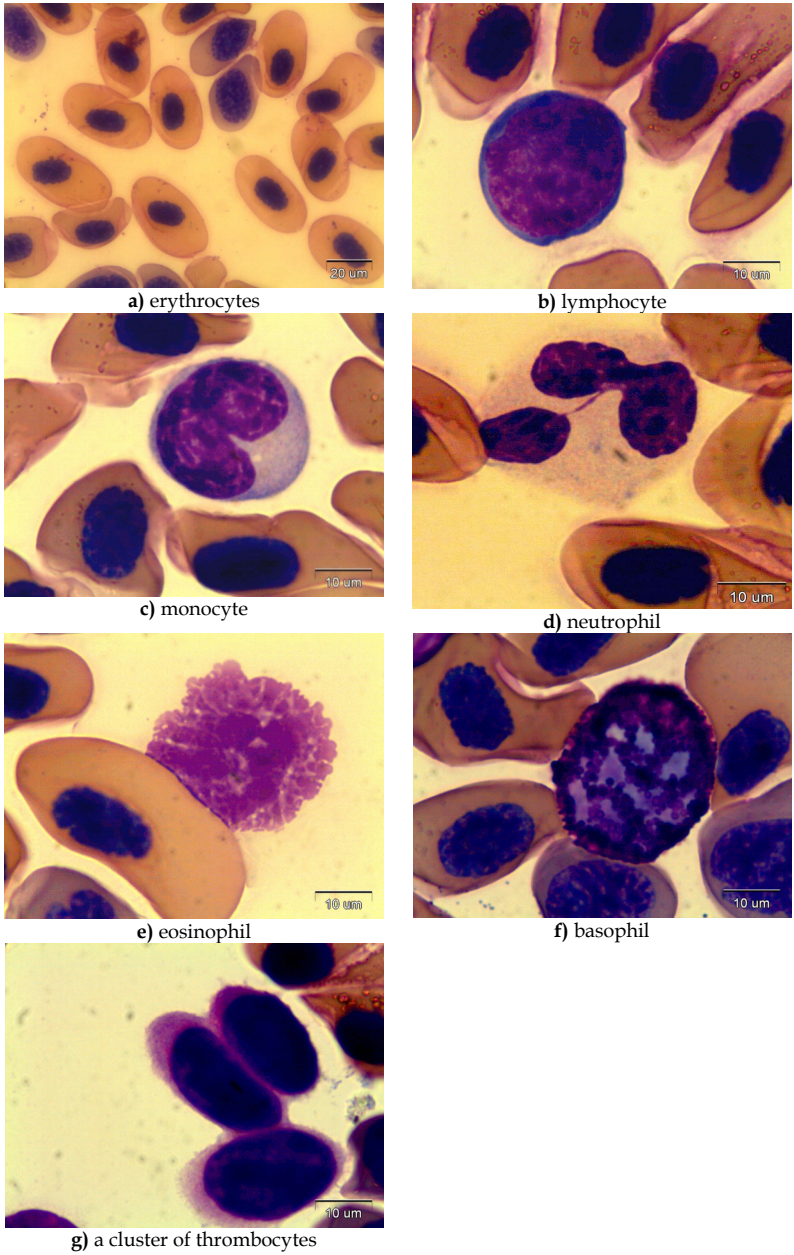
The mean diameter of the eosinophils was measured as 26.38 µm (Table 2). Their cytoplasm stained a light blue, and nuclei dark blue. Large, roundish, bright reddish granules characterized their cytoplasm (Fig.1e). Nuclei were usually bilobed. They were scarcer than the neutrophils.

The mean diameter of the few observed basophiles was 17.01 µm (Table 2). Using Wright's stain; their cytoplasm appeared light blue with dark purple to blue granules partly obscuring the dark blue nucleus (Fig.1f). Basophiles were the scarcest leucocytes.

Finally, the spindle shaped thrombocytes had a mean length of 20.02 µm and a mean width of 12.17 µm (Fig.1g, Table 2). With Wright's stain, the cytoplasm was coloured pale blue and their nuclei were dark purple. The darkly stained large oval nuclei left a small, irregular cytoplasmic area. They tended to clump together in blood smears.

Discussion

The number of erythrocytes apparently is regulated by several feedback mechanisms that work with much greater precision in



a) erythrocytes
b) lymphocyte
c) monocyte
d) neutrophil
e) eosinophil
f) basophil
g) a cluster of thrombocytes

Figure 1. Blood cells of the Lycian salamander

Table 2. Established measurements (μm) and sizes (μm²) concerning blood cells of Lycian salamander. [n: number of specimens, SD: standard deviation, L: erythrocyte length, W: erythrocyte width, S: erythrocyte size, NL: nucleus length, NW: nucleus width, NS: nucleus size, N/C: nucleocytoplasmic ratio, TL: thrombocyte length, TW: thrombocyte width]

Characters	Sex	n	Mean	SD	Range
L (μm)	♂♂	5	39.59	2.096	33.00 – 44.00
	♀♀	5	37.67	2.134	32.00 – 45.00
	♂♀	10	38.63	2.322	32.00 – 45.00
W (μm)	♂♂	5	20.32	1.531	16.00 – 24.00
	♀♀	5	19.45	1.688	16.00 – 25.00
	♂♀	10	19.88	1.668	16.00 – 25.00
L/W	♂♂	5	1.96	0.169	1.50 – 2.47
	♀♀	5	1.95	0.177	1.45 – 2.38
	♂♀	10	1.95	0.173	1.45 – 2.47
S (μm²)	♂♂	5	631.89	63.125	450.20 – 794.40
	♀♀	5	575.60	66.455	440.40 – 812.50
	♂♀	10	603.74	70.597	440.40 – 812.50
NL (μm)	♂♂	5	16.68	1.010	14.00 – 20.00
	♀♀	5	16.53	1.153	14.00 – 19.00
	♂♀	10	16.60	1.085	14.00 – 20.00
NW (μm)	♂♂	5	9.63	0.804	8.00 – 12.00
	♀♀	5	9.99	0.720	8.00 – 13.00
	♂♀	10	9.81	0.784	8.00 – 13.00
NL/NW	♂♂	5	1.74	0.143	1.40 – 2.20
	♀♀	5	1.66	0.123	1.20 – 2.00
	♂♀	10	1.70	0.140	1.20 – 2.20
NS (μm²)	♂♂	5	126.34	15.390	94.20 – 188.40
	♀♀	5	129.90	15.605	93.40 – 179.00
	♂♀	10	128.12	15.580	93.40 – 188.40
N/C	♂♂	5	5.05	0.622	3.48 – 7.55
	♀♀	5	4.46	0.446	3.42 – 5.93
	♂♀	10	4.75	0.617	3.42 – 7.55
Small Lymphocytes (μm)	♂♂	5	18.06	1.824	15.00 – 22.50
	♀♀	5	18.44	1.562	15.00 – 22.50
	♂♀	10	18.26	1.704	15.00 – 22.50
Large Lymphocytes (μm)	♂♂	5	26.20	1.921	22.50 – 32.25
	♀♀	5	25.82	2.148	22.50 – 32.50
	♂♀	10	26.01	2.041	22.50 – 32.50

Table 2. (Continued

Characters	Sex	n	Mean	SD	Range
	♂♂	5	29.03	2.472	25.00 – 38.00
Monocytes (µm)	♀♀	5	30.62	2.497	25.00 – 38.00
	♂♀	10	29.83	2.605	25.00 – 38.00
	♂♂	5	26.79	2.612	21.20 – 32.50
Neutrophils (µm)	♀♀	5	27.30	2.224	22.50 – 32.50
	♂♀	10	27.04	2.433	21.20 – 32.50
	♂♂	5	25.92	2.917	20.00 – 32.50
Eosinophils (µm)	♀♀	5	26.84	2.496	21.20 – 32.50
	♂♀	10	26.38	2.747	20.00 – 32.50
	♂♂	5	16.32	1.775	12.50 – 22.50
Basophils (µm)	♀♀	5	17.70	2.360	15.00 – 25.00
	♂♀	10	17.01	2.189	12.50 – 25.00
	♂♂	5	19.77	2.751	12.50 – 25.00
TL (µm)	♀♀	5	20.25	3.711	13.80 – 30.00
	♂♀	10	20.02	3.259	12.50 – 30.00
	♂♂	5	11.67	1.089	10.00 – 13.80
TW (µm)	♀♀	5	12.67	1.675	10.00 – 16.20
	♂♀	10	12.17	1.493	10.00 – 16.20

animals with a constant body temperature and high tissue metabolism than in amphibians and reptiles (Hutchinson & Szarski 1965). The number of erythrocytes in amphibian circulating blood shows a wide individual variation and considerable interpecific differences (Hutchison & Szarski 1965, Szarski & Czopek 1966), as well as depending on weight, age, sex, habitat conditions and season (e.g. Sinha 1983, Ruiz et al. 1989, Wojtaszek et al. 1997). In the present study, the erythrocyte count was lower than that of the other urodele species listed in Table 3. However, Hutchinson & Szarski (1965) estimated the red blood cells of the rough skinned newt, *Taricha granuose*, was $1.11x10^5$ ($0.70x10^5$ – $1.90x10^5$)/mm^3. Furthermore, Friedmann et al. (2005) calcuated $0.40x10^5$/mm^3 and a haemoglobin conent of 4.5 g/dl in the same species.

Urodeles show the lowest erythrocyte counts and degree of vascularization, ranid frogs appear be intermediate, while species of *Bufo* and *Hyla* show the greatest degree of vascularization and also have the highest erythrocyte counts Erythrocyte counts were $6.15x10^5$ ($3.00x10^5$ – $9.00x10^5$)/mm^3 and $6.58x10^5$ ($4.00x10^5$ – $8.41x10^5$)/mm^3 for *Hyla versicolor* and *Bufo americanus*, respectively (Hutchinson & Szarski 1965). Rouf (1969) estimated that the erythrocyte count of *Rana pipiens* was $1.20x10^5$ – $4.70x10^5$ (mean, $3.19x10^5$)/mm^3. In addition, Alder & Huber (1923) calculated that the red blood cells of *Rana temporaria* were $4.08x10^5$/mm^3. The erythrocytes of the Lycian salamander

Table 3. Number and size of erythrocytes in some urodele species.

Species	Erythrocyte Count (per mm^3)	Erythrocyte Size (in μm^2)
Lyciasalamandra fazilae	$1.06x10^5/mm^3$ (Present study)	603.74 (Present study)
Lyciasalamandra atifi		507.54 ** (Atatür et al. 1998)
Mertensiella caucasica		440.44 **
Salamandra salamandra		523.44 **
Lissotriton vulgaris	$1.74x10^5/mm^3$ **** (Tosunoğlu et al. 2008)	421.35 ****
	$1.98x10^5/mm^3$ * (Szarski & Czopek 1966)	419.44 **
Triturus karelinii	$2.28x10^5/mm^3$ *	420.37 **
Ommatotriton vittatus		367.05 **
Neurergus strauchii	$1.27x10^5/mm^3$ *** (Arıkan et al. 2003)	463.82 ***

($1.06x10^5/mm^3$) from our results were 3 – 4 times lower than that of *R. pipiens* and *R. temporaria*, and almost 6 times lower than that of *Hyla versicolor* and *Bufo americanus*.

The leucocyte count varies depending on species, season, sex, nutritional conditions and some physiological conditions (e.g. diseases, breeding) (Rouf 1969, Arıkan 1989, Wojtaszek & Adamowicz 2003). In the Lycian salamander, the number of leucocytes displays a wide range between $1.60x10^3$ and $2.87x10^3/mm^3$. We found sexual dimorphism between sexes. Female leucocytes number slightly higher than males. Most probably this is related to the physiological condition (e.g. weight, age, sex) of individuals.

Erythrocytes are morphologically similar among various species of amphibians. According to Wintrobe (1933), the erythrocyte size reflects the position of a species on the evolutionary scale: in lower vertebrates and those with a not so successful evolutionary past, i.e. in cyclostomes, elasmobranches and urodeles, the erythrocytes are large, but in higher vertebrates (mammals) the same cells are smaller and do not contain nuclei. According to Evans (1939), a correlation was found between erythrocyte size and activity level in salamanders. Several authors (Haden 1940, Altman & Dittmer 1961, Harris 1963, Atatür et al. 1998) pointed out that various environmental factors and activity levels affect erythrocyte size. In this study, erythrocytes showed substantial variation in size and number (Table 1). Our results show that the erythrocyte size of *L. fazilae* was bigger than

previous studies have found for *Salamandra salamandra*, *Mertensiella caucasica*, *Lissotriton vulgaris*, *Ommatotriton vittatus* and *Triturus karelinii* (Table 3).

Atatür et al. (1999) found that aquatic anurans have larger erythrocytes than semi-aquatic and terrestrial species. Aquatic and semi-aquatic urodeles (*Lissotriton vulgaris, Triturus karelinii*, *Ommatotriton vittatus*, *Neurergus strauchii*, and *Mertensiella caucasica*) have smaller erythrocytes than terrestrial ones (*Lyciasalamandra fazilae*, *Lyciasalamandra atifi*, *Salamandra salamandra*) (Table 3).

In conclusion, the hematology of the Lycian salamander was similar to other urodele species mentioned in the literature. Although small differences were observed between sexes in terms of these values, some were statistically significant. These differences probably arose from individual variation, physiological conditions, and the other parameters discussed above. Even though small differences were observed in terms of cell sizes, they were also concluded to be individual variations.

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