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Immunohematological Parameters of Ectothermal Amphibians of the Fauna of the Middle Urals: Siberian Salamander *Salamandrella Keyserlingii* Dybovsky, 1870 (Caudata) and Lake Frog *Pelophylax Ridibundus* Pallas, 1771 (Anura)

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Abstract—A comparative analysis of hematological parameters was for the first time performed in two ectothermal amphibians of the Middle Ural fauna, the Siberian salamander *Salamandrella keyserlingii* and lake frog *Pelophylax ridibundus*. Species specificity of immune defense was demonstrated with respect to granulocyte and agranulocyte counts ($p < 0.001$). A high lymphocyte content (73.3–76.1%) of provides for the activation of acquired adaptive immunity mechanisms in the thermophilic lake frog. The Siberian salamander is adapted to low negative temperatures and has a set of nonspecific leukocytes (39.3–44.4%). Innate immunity is better developed in the Siberian salamander compared with the lake frog.

Keywords: Siberian salamander, lake frog, lymphocytes

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Amphibian population sizes and diversity are decreasing to impair the stability of aquatic and terrestrial communities in various regions of the globe [1–3]. Two amphibians differing in temperature tolerance inhabit both natural and anthropogenic landscapes of the Ural mountains: the lake frog (LF) *Pelophylax ridibundus* Pallas, 1771 of the order Anura and the Siberian salamander (SS) *Salamandrella keyserlingii* Dybovsky, 1870 of the order Caudata [4]. We have previously studied the mechanisms of homeostasis in SS and LF and observed species-specific features in their thermostable amino acid spectra of the blood plasma, the features contributing to their survival and euribiontness in a broad temperature range [5]. Free amino acids that possess immunomodulatory properties and are involved in regulating hematopoiesis were additionally detected in the blood [5]. Evolutionarily, the vertebrate blood system is known to play a crucial role in the development of resistance to various biotic and abiotic environmental factors (temperature, hypoxia, toxic chemicals, parasite invasions, and stress) [6–8]. The blood system is highly organized in herptiles, and the composition and morphology of their lymphoid system do not differ from those reported from vertebrate studies [9–14]. This makes it possible to think that the SS adaptive strategy of remaining at extremely low temperatures (–30 to –45°C) for a long period of time and LF tolerance to extremely high temperatures (28–40°C) certainly determine the survival of the amphibians [5, 15, 16]. However, there is only limited data on immune defense mechanisms working in natural populations of tailed and tailless amphibians, and nearly nothing is known on their adaptive immune reactions and the role of nonspecific immune responses in regulating physiological processes [6].

The objective of this work was to compare the immunohematological parameters of the peripheral blood for two ectothermal amphibians from natural populations of the Middle Ural fauna, the SS *S. кeyserlingii* and LF *P. ridibundus*.

Study samples included adult males of *S. кeyserlingii* ($n = 20$) and *P. ridibundus* ($n = 20$), which were captured in Sverdlovskaya oblast (56°42′ N, 61°20′ E) in the spring (the first decade of May) and summer (the last decade of July) seasons of 2019 and 2020. Amphibians were captured with a dip net from spawning water bodies; summer SS samples were collected manually from terrestrial shelters. Amphibians without signs of disease were individually placed in containers with wet moss and transferred to the lab the same day. The SS *S. кeyserlingii* is in the Red List of the Middle Urals [17] and has a category III status. The species is rare and has lower population sizes at the periphery of its range. Its representatives are found ubiquitously, but sporadically. Study samples were collected in shaded near-bank areas of the Kalinovskii Pond, which is in a specially protected parkland zone

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of an urban complex. The water temperatures were 11°C in spring and 17°C in summer; the mean daily air temperatures were 15 and 21°C, respectively. Adult SSs live on the ground near the bank of a spawning water body throughout their lives except for a short reproduction period. SSs do not tolerate prolonged exposure to sunlight and die at a shade air temperature of approximately 27°C. It should be noted that the majority of amphibian species are incapable of adapting to warm waters, while LF (which is an invasive species in the Urals) has been found to inhabit thermal water bodies with a water temperature reaching 30°C [4]. LF study samples were collected in shallow backwaters of the Tagil River, where the water temperatures are 21°C in spring and 27°C or higher in summer on average and the mean daily air temperatures are 14 and 19°C, respectively. The water temperature does not decrease below 10–12°C in winter, and LFs living in the river do not hibernate. Peripheral blood parameters were assayed using a BC-5800 hematology analyzer (Mindray, China). A differential blood count was obtained by examining 100 white blood cells in blood smears stained with a Romanowsky–Giemsa stain. The differential blood count was used to calculate the integral leukocyte shift index (LSI) as a granulocyte to agranulocyte ratio (relative units), which characterizes modulation of effector mechanisms of the immune system and the tension of compensatory processes in the body. The results were processed using the software package Statistica v. 10.0. A principal component analysis (PCA) was carried out in the R statistical environment R (R 3.1.2, packages Vegan and Ade4) [18].

Peripheral blood assays showed that the hemoglobin content in SS (59.8 ± 3.3 g/L) is significantly, 1.5 times, higher than in LF (40.2 ± 2.5 g/L) ($p = 0.04$). The hemoglobin contents agreed with estimates reported by other Russian researchers [10, 14, 19]. A 3.5 times higher platelet content (41.7 ± 5.8 g/L) ($p = 0.01$) and a twice higher platelet crit (PCT = 0.02%) in LF compared with SS were associated with a higher whole blood volume portion of platelets, which are involved in immune responses. High leukocyte contents (77.0 ± 7.5 g/L) were observed in SS in spring and summer, being 2.4 times higher than in LF ($p = 0.001$). Like in all vertebrates, two cell groups were observed in LF and SS leukocyte populations: granulocytes (neutrophils, eosinophils, and basophils), which determine innate immunity reactions, and agranulocytes (monocytes and lymphocytes), which are responsible for adaptive immune responses [6, 20]. A differential blood count of the peripheral blood showed a lymphoid-shifted profile in both amphibians in spring and summer; the lymphocyte contents were 69.1–69.8% in LF and 52.4–56.7% in SS (Table 1). Species-specific differences were observed in lymphocyte, granulocyte, and agranulocyte counts, which reflect the relationship of effector mechanisms of the immune system in the genetically determined amphibian species ($p < 0.001$) (Table 1).

Agranulocytes, which are responsible for immune surveillance and selective responses of the body, predominated in the peripheral blood in both of the species; their proportions were 73.3–76.1% in LF and 55.7–60.3% in SS ($p < 0.001$). The lymphocyte–granulocyte composition of the peripheral blood showed a lower granulocyte portion in LF (24.0–26.7%) as compared with SS (39.3–44.4%) ($p < 0.001$). The granulocyte proportion in the peripheral blood of SS was 1.7 times higher than in LF in spring and summer ($p < 0.001$), while the agranulocyte proportion in LF was 30% higher than in SS (Table 1). A higher neutrophil level in the peripheral blood of SS ($p < 0.001$) ensured nonspecific protection from toxicants and pathogen infections. Opposite seasonal changes in band neutrophils were observed in LF and SS ($p < 0.001$) (Table 1). Segmented neutrophil contents decreased in summer by a factor of 2.7 in LF and 3.3 in SS.

Eosinophil granulocytopoiesis increased in summer by a factor of 2.6 in LF ($p < 0.001$) and 1.6 in SS ($p < 0.001$), indicating that common mechanisms determine the adaptive strategies of antimicrobial and antihelminthic immune defense in animals [13, 14]. Basophils, which are involved in cell-mediated inflammatory reactions, occurred in considerable amounts (0.08–0.09%) in the blood of adult SS males in spring and summer and were absent in the LF blood. Interspecific differences in monocyte content were undetectable in spring ($p = 0.15$), but were observed in summer ($p < 0.001$). Activated monocytes are known to play a phagocytic role and to produce anti-inflammatory cytokines, which act as endogenous regulators of hematopoiesis and cell-mediated immune responses [7, 11, 20].

It seems that monocytes increase the production of proinflammatory cytokines in response to environmental endotoxins in summer and thus activate the natural immunity system in LF in contrast to SS ($p < 0.001$). LSI calculations confirmed the interspecific differences in leukocyte profile between the species under study. The LSI of SS (0.65–0.80) was higher than that of LF (0.32–0.36), testifying again to a higher reactivity of innate immunity, which ensures nonspecific immediate defense against environmental pathogenic antigens ($p < 0.001$). Thus, there are common features in the mechanisms that maintain homeostasis in the two amphibians, LF and SS, as well as certain strategic differences in the mechanisms that are responsible for the emergency regulation of immunity.

PCA was used to visualize the species- and season-specifics of leukocyte compositions in SS and LF and confirmed the results of the above statistical analysis. Principal component 1 (PC1) accounted for 46.93% of the total variance of blood parameters; principal component 2 (PC2), for 31.92% of the total variance (Fig. 1). PC1 and PC2 together determined the signif-

Table 1. Differential blood count parameters in LF *P. ridibundus* and SS *S. keyserlingii* males: arichmetic mean ($X_{M\ \mathrm{boot}}$), error of the mean (SE_{boot}), and 95% confidence interval ([95% CI_{boot}]) of a bootstrap distribution

Parameter, %	Species	I. Spring LF ($n = 8$) SS ($n = 11$)	II. Summer LF ($n = 12$) SS ($n = 9$)	Tukey's test (p-value)	
Neutrophils	LF	24.1 ± 1.4 [21.1–26.8]	17.1 ± 0.5* [16.2–18.0]	I–II (<0.001)	Species I: <0.001 II: <0.001
	SS	40.6 ± 1.0@ [38.8–42.6]	33.4 ± 0.6*@ [32.3–34.4]	I–II (<0.001)	
– early	LF	3.3 ± 0.3 [2.6–4.0]	5.8 ± 0.4* [5.2–6.5]	I–II (<0.001)	Species I: <0.001 II: <0.001
	SS	6.2 ± 0.3@ [5.7–6.7]	9.3 ± 0.4*@ [8.6–10.0]	I–II (<0.001)	
– band	LF	9.2 ± 0.7 [7.9–10.5]	6.9 ± 0.6* [5.8–8.0]	I–II (0.05)	Species I: 0.28 II: <0.001
	SS	10.1 ± 0.4 [9.3–10.9]	17.1 ± 0.3*@ [16.4–17.8]	I–II (<0.001)	
– segments	LF	11.5 ± 1.0 [9.7–13.7]	4.2 ± 0.2* [3.7–4.6]	I–II (<0.001)	Species I: <0.001 II: <0.001
	SS	23.8 ± 0.6@ [22.7–24.9]	7.1 ± 0.5*@ [6.1–8.0]	I–II (<0.001)	
Eosinophils	LF	2.7 ± 0.3 [2.1–3.2]	6.9 ± 0.5* [5.9–7.9]	I–II (<0.001)	Species I: 0.05 II: 0.10
	SS	3.8 ± 0.2@ [3.4–4.3]	5.9 ± 0.3* [5.4–6.3]	I–II (<0.001)	
Monocytes	LF	4.2 ± 0.6 [3.0–5.4]	6.3 ± 0.5* [5.4–7.3]	I–II (0.001)	Species I: 0.15 II: <0.001
	SS	3.3 ± 0.2 [2.9–3.7]	3.6 ± 0.2@ [3.3–3.9]	I–II (0.60)	
Lymphocytes	LF	69.1 ± 0.9 [67.5–71.1]	69.8 ± 0.5 [68.8–70.8]	I–II (0.60)	Species I: <0.001 II: <0.001
	SS	52.4 ± 1.0@ [50.4–54.4]	56.7 ± 0.8*@ [55.2–58.1]	I–II (0.001)	

Differences (*) between seasons I and II or (@) between the species were significant at $p < 0.05$. LF, lake frog; SS, Siberian salamander.

icant differences in main leukocyte parameters between the two amphibians. Based on PC1, the highest contributions to the interspecific variation of blood parameters are made by lymphocytes (30.43%), monocytes (25.01%), band neutrophils (17.68%), and segmented neutrophils (16.52%). Their coefficients of correlation with PC1 were, respectively, 0.93, 0.84, –0.71, and –0.68 ($p < 0.001$). Based on these variables, PC1 isolated both individuals of cold-resistant SS and those of heat-resistant LF into distinct groups (Fig. 1). PC2 highly correlated with eosinophils (0.80), early neutrophils (0.81), and segmented neutrophils (–0.62). These parameters contributed 33.67, 34.18, and 20.29%, respectively, to the seasonal variation of the differential blood count in the two amphibians, indicating again that individuals are heterogeneous with respect to the parameters in the two ecologically distinct amphibian species. As is seen from Fig. 1, the data clustered into four separate groups, and their greatest spatial differentiation was due to seasonal alterations in the blood leukocyte composition.

To summarize, we were the first to evaluate the species specificity of effector mechanisms of the immune system in regulating physiological processes of the amphibians LF and SS. The species-specific features contribute to the survival and euribiontness of the two species in a broad temperature range in natural and anthropogenic landscapes of the Middle Urals. Knowledge of genetically determined immunohematological parameters of homeostasis of species is of theoretical interest and applied significance for developing nature protection measures in conditions of rapid environmental changes.

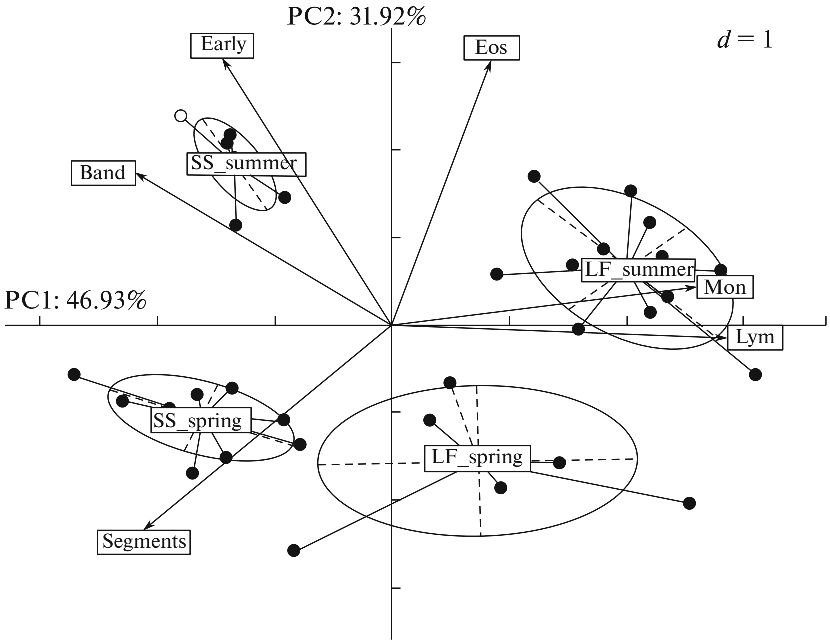

Fig. 1. PCA of differential blood count (%) in SS and LF in different seasons. PC1 and PC2 are axes of principal components 1 and 2, respectively; %, percent total variance explained by the respective principal component. Arrows show the correlations of PC1 and PC2 with initial parameters; ellipses show the 95% confidence areas.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. Animals were captured and kept in the lab in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, 1986.

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