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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Amino Acid Spectrum in the Blood of the Endemic and Invasive Amphibian Species in the Fauna of the Ural

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Abstract—For the first time, the results of a comparative analysis of free amino acids in the blood plasma of amphibians of the Ural fauna are presented: an endemic species (Siberian salamander (*Salamandrella keyserlingii* Dybovsky, 1870)) and an invasive species (marsh frog (*Pelophylax ridibundus* Pallas, 1771)). The species diversity of adaptive strategies of thermoresistant amphibionts both to negative and positive temperatures is shown.

Keywords: Siberian salamander, marsh frog, amino acids, blood

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Amphibians as one of the dominant groups among all classes of vertebrates make a significant contribution to the species diversity of not only aquatic but also terrestrial ecosystems. Currently, about a third of the species of these animals are under the threat of extinction [1, 2]. Representatives of the Ural amphibian fauna from the order Caudata (the Siberian salamander *Salamandrella keyserlingii* Dybovsky, 1870) and from the order Anura (the marsh frog *Pelophylax ridibundus* Pallas, 1771) are of great interest, because they have multidirectional adaptive strategies as poikilothermic amphibionts dwelling in the range of extreme positive and negative temperatures [3, 4]. Researchers noted an increased cold resistance of male and female Siberian salamanders and their ability to tolerate temperatures below –35 to –40°C without losing activity at 0 to 2°C [5, 6]. The strategy of existence of the Siberian salamander under conditions of low positive and even negative temperatures contributes to the possibility of expanding its range, the northern boundary of which runs beyond the Arctic Circle. Animals do not tolerate long-term exposure to the sun and die at temperatures in the shadow approximately 27°C. However, the marsh frog, an invasive species for the Urals, is tolerant to high positive temperatures. In the Urals, this species initially inhabited thermal reservoirs of anthropogenic origin (cooling reservoirs of the Verkhnetagilskaya and Reftinskaya hidroelectric power stations, Sverdlovsk region). It is known to inhabit hot springs, where the water temperature reaches 30°C and sometimes even higher [4, 7]. Amino acids (AAs) and their derivatives, as universal biological modifiers and regulators of the key metabolic reactions, are responsible for maintaining the biological integrity of the organism [3]. It is known that free AAs have a regulatory role in the formation of the adaptive strategy of animals, which ensures the stability of population homeostasis under conditions of both aquatic and terrestrial microclimate [8–11]. However, information on the metabolism of free AAs in the blood plasma of amphibians of the Ural fauna is missing in available literature. This is the first study to analyze the content of free AAs in the blood plasma of sexually mature male amphibians ($n = 11$) dwelling in spawning water bodies and in coastal ground shelters at an average daily air temperature of 23°C on the territory of the Sverdlovsk region (58°0′0″ N, 62°0′0″ E). The climate of the study area is continental with long cold winters and relatively warm short summers. The typical invasive species, marsh frog, was caught in shallow backwaters of the Tagil River. In summer, the water temperature averages 27.5 ± 0.4°C. In the period of catching salamanders in the shore zone of Kalinovskii Pond in the forest park of the urban agglomeration of Yekaterinburg, the water temperature averaged 21 ± 0.3°C. Animals were brought to the laboratory on the day of capture in July–August 2019. Animal blood samples were taken from the myocardium. Blood plasma was obtained by centrifugation of blood in a K-23D refrigerated ultracentrifuge (Germany) in Bekton Dickinson BP vacutainers (UK) with EDTA for 15 min at 3000 rpm. The content of free AAs in the blood plasma of animals was determined by high performance liquid chromatography on an Agilent 1260 Infinity II analyzer (Germany). The concentration of AAs was expressed in μmol/L and as a percentage of the total content. In total, 187 amino acid samples were ana-

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Table 1. Free amino acid content (μmol/L) in the blood plasma of male *S. keyserlingii* and *P. ridibundus*. Data are presented as the mean values (*m*), standard errors (*SE*), and confidence intervals (95% CI)

AA	Siberian salamander	Marsh frog
Histidine	188.9 ± 5.1 [174.9–203.1]*	80.2 ± 5.9 [65.1–95.3]
Threonine	35.4 ± 0.8 [33.3–37.6]	70.3 ± 6.0 [54.9–85.7]*
Arginine	78.6 ± 4.0 [67.5–89.8]*	38.6 ± 1.6 [34.5–42.6]
Valine	31.9 ± 0.9 [29.6–34.3]	81.9 ± 3.9 [71.6–92.1]*
Methionine	72.4 ± 2.3 [66.3–78.6]*	18.4 ± 2.8 [11.3–25.5]
Phenylalanine	27.4 ± 0.9 [24.9–29.8]	49.1 ± 3.0 [41.3–56.9]*
Isoleucine	7.7 ± 0.2 [7.2–8.2]	61.5 ± 2.6 [54.7–68.2]*
Leucine	33.8 ± 1.1 [30.9–36.8]	221.2 ± 12.4 [189.3–253.0]*
Lysine	103.3 ± 1.2 [99.9-106.6]	195.3 ± 7.3 [176.7–213.9]*
EAA	579.6 ± 5.8 [563.4–595.7]	816.4 ± 19.5 [766.3–866.4]*
Free AA pool	1086.1 ± 15.4 [1043.2–1128.9]	1470.4 ± 49.0 [1344.0–1596.3]*

* Statistically significant differences between groups, Tukey test (ANOVA), $p < 0.0001$; EAA—essential amino acids.

lyzed. The results were processed using the Statistica v.10.0 licensed software package. Principal component analysis (PCA) was implemented using the R statistical environment (R 3.1.2, Vegan and Ade4 packages) [12].

The amino acid spectrum of amphibian blood plasma is represented by 17 AAs: aspartate, glutamate, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, cysteine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, and proline. Comparative analysis showed significant interspecies differences in the pool of free AAs in blood plasma: 1470.4 ± 49.0 μmol/L in the marsh frog and 1086.1 ± 15.4 μmol/L in the Siberian salamander ($p < 0.0001$). The tested amphibians, similarly to homeothermic animals, contained the full range of functionally important essential Aas (threonine, valine, lysine, leucine, isoleucine, methionine, phenylalanine, arginine, and histidine) (Table 1). It should be noted that essential AAs (EAAs) cannot be obtained as a result of biosynthesis and must enter the body in the form of food proteins from the outside, and the absence of EAAs entails life-threatening phenomena [9]. Noteworthy is the high percentage of EAAs in the blood of the Siberian salamander and marsh frog (53.4 and 55.6%, respectively, of the total AA pool). According to researchers, the coefficient of the essential-to-nonessential AA ratio indicates the balance of nitrogen and protein metabolism in amphibians [8–10]. A consistently high amino acid balance in blood plasma of both the Siberian salamander ($C_{EAA/NEAA} = 1.15$) and marsh frog ($C_{EAA/NEAA} = 1.25$) was noted.

The amino acid spectrum of the blood plasma of amphibionts reflects not only energy metabolism and involvement in protein synthesis but also its adaptive role in evolutionarily fixed processes with variability of a wide range of temperatures of the habitat. The dominant AAs in the blood plasma of the Siberian salamander are aspartate, cysteine, arginine, and histidine; their total content is 48% of the total pool, which is 3.6 times higher than in the marsh frog. The high concentration of glycogenic aspartate in the blood plasma of the Siberian salamander also indicates its role as a low-temperature adaptogen for this species [14]. The greatest contribution to the pool of essential AAs in the Siberian salamander is made by the amino acids whose frequency of excess in comparison with the marsh frog was 2.4 for histidine, 2.0 for arginine, and 3.9 for methionine. The high total content of methionine and cysteine as immunomodulators in the blood plasma of the Siberian salamander (177.6 μmol/L) should also be noted, which is an order of magnitude higher than the content of these AAs (16.3 μmol/L) in the marsh frog.

Our findings, as well as the published data, suggest that the significant accumulation of these amino acids in the blood plasma of the Siberian salamander contributes to the protection of cell membranes from functional destabilization and possibly suggests their protective role at low temperatures [10, 13–15].

In the heat-resistant marsh frog, the total content of the main glycogenic AAs alanine (16.5%), glutamate (6.2%), and glycine (9.3%) of the total AA pool is 32%, which is 2.0 times higher than in the Siberian salamander. A specific feature of the frog AA pool, according to our results, is the significant concentrations of leucine and lysine (up to 15.1 and 13.3%, respectively, of the total pool). The predominant accumulation of these AAs suggests that their high content, due to involvement in energy exchange processes, is also suggestive of their special reserve role in ensuring the stability and tolerance of frogs to near-zero temperatures [11, 14, 15].

The principal component method was used to visualize the species specificity of the essential amino acids in blood plasma of the Siberian salamander *S. keyser-*

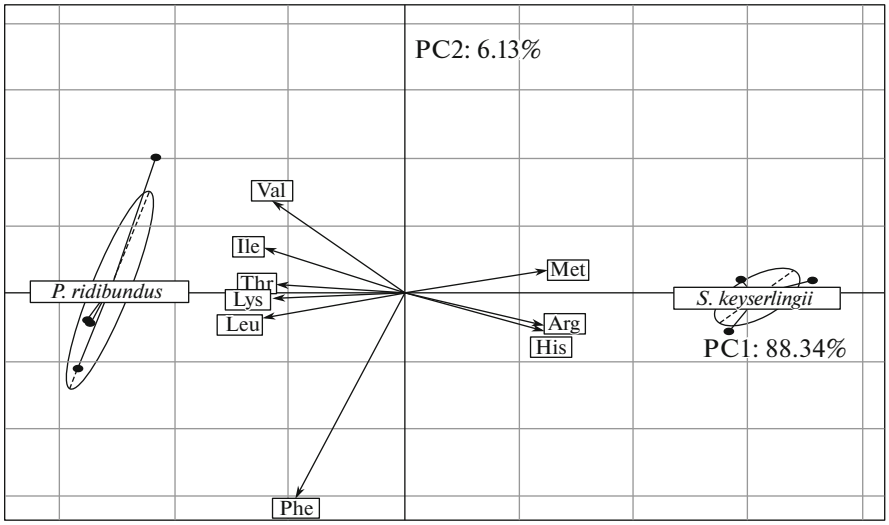

Fig. 1. Content of essential amino acids (% of the AA stock) in the blood plasma of male Siberian salamanders *S. keyserlingii* and marsh frog *P. ridibundus* in the space of the principal components. Designations: PC1, PC2—principal component axes, %—percent of data variance explained by the principal component; arrows show the correlation of the principal components with the initial values (amino acids); ellipses represent 95% confidence areas.

lingii and the marsh frog *P. ridibundus*. For the first principal component (PC1), which accounts for 88.34% of the total variance of the data, a significant spatial differentiation of individuals of the marsh frog and Siberian salamander according by the percentage of essential AAs in blood plasma was shown (Fig. 1). The highest correlations with PC1 were found for histidine (0.98), arginine (0.98), and methionine (0.99), especially high blood levels of which are typical for the Siberian salamander, as well as isoleucine (–0.98) and leucine (–0.99) for the marsh frog ($p < 0.001$). The contribution of each of these essential amino acids to interspecies differences exceeds 12.03% (Table 2).

Phenylalanine makes the smallest contribution to the total data variance (7.24%); its correlation with PC1 was –0.76 ($p < 0.05$). For the percentage of this

Table 2. Results of the component analysis of essential amino acids (% of the AA pool) in the blood plasma of male Siberian salamanders and marsh frogs (correlation coefficients between nine blood plasma amino acids and the principal components PC1 and PC2 (use of the Ade4 package)

AA, % ($i = 9$)	Loadings, a_{ij}		Contribution to principal component (contribution = $(a_{ij}^2 \times 100)/\lambda_j$, %)	
	principal components (PC), $j = 1, 2$			
	1	2	1	2
Histidine	0.98***	–0.12	12.17	2.52
Threonine	–0.89**	0.03	10.04	0.14
Arginine	0.98***	–0.10	12.12	1.82
Valine	–0.92***	0.29	10.71	15.25
Methionine	0.99***	0.07	12.22	0.84
Phenylalanine	–0.76*	–0.64	7.24	74.71
Isoleucine	–0.98***	0.14	12.04	3.62
Leucine	–0.99***	–0.08	12.27	1.04
Lysine	–0.94***	–0.02	11.19	0.06
	Eigenvalues (λ_j) PC		PC-explained variance (%)	
	7.95	0.55	88.34	6.13

Correlation coefficients were statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

aromatic amino acid in the blood of the studied groups of amphibians, the smallest though statistically significant interspecific differences were found ($p < 0.01$).

Thus, the content of free amino acids in the blood plasma of amphibians of the Ural fauna—the Siberian salamander (endemic species) and the marsh frog (invasive species)—was studied for the first time. The results obtained make it possible to assess the species specificity of the amino acid spectrum, which ensures the survival and eurybionism of the studied species in a wide temperature range.

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COMPLIANCE WITH ETHICAL STANDARDS

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

Statement on the welfare of animals. The capture and maintenance of animals in the laboratory was carried out in accordance with the rules adopted by the European Convention for the Protection of Animals used for Experimental and Scientific Purposes (*European Convention*, 1986).

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