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COMPARATIVE AND ONTOGENIC BIOCHEMISTRY

Free Amino Acid Profile in Blood Plasma of Bats (*Myotis dasycneme* Boie, 1825) Exposed to Low Positive and Near-Zero Temperatures

L. A. Kovalchuk[a]*, V. A. Mishchenko[a, b], N. V. Mikshevich[c], L. V. Chernaya[a], M. V. Chibiryak[a], and A. P. Yastrebov[d]

[a] *Institute of Plant and Animal Ecology, Ural Branch of the Russian Academy of Sciences, Yekaterinburg, Russia*

[b] *Ural Federal University named after the first President of Russia B. N. Eltsin, Yekaterinburg, Russia*

[c] *Ural State Pedagogical University, Yekaterinburg, Russia*

[d] *Ural State Medical University, Yekaterinburg, Russia*
**e-mail: kovalchuk@ipae.uran.ru*

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Abstract—We analyze here for the first time the plasma free amino acid profile in pond bats (*Myotis dasycneme* Boie, 1825) living in the boreal Ural region and exposed experimentally to low positive and near-zero temperatures during their preparation for hibernation. Pond bats were caught in their mass habitation territory in the Middle Ural near the Smolinsky cave (N 56°28', E 61°37') in the third decade of September 2015. Qualitatively, blood plasma in pond bats contains 21 amino acids. In a model experiment carried out on pre-hibernating animals at a regular hibernation temperature (0–2°C), the total plasma pool of free amino acids increased significantly by 42% (irrespective of sex) and reached 1561.4 ± 112.6 μmol/L ($p = 0.01$). Under these experimental conditions, the fraction of glucogenic amino acids rose by 34% ($p = 0.01$) and that of essential ones by 80% ($p = 0.001$). Both in control and experimentally cooled pre-hibernating animals, the plasma was found to lack tryptophan, suggesting its utilization as a substrate in the synthesis of serotonin, a biogenic amine directly involved in the maintenance of hypothermia and hypometabolism in these chiropterans.

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INTRODUCTION

Studying the physiological and biochemical mechanisms that underlie cold tolerance provides better insight into the evolution and ecology of heterothermic animals [1, 2]. Some authors believe that, while temporal heterothermy is an important survival mechanism, most of recently extinct mammals may have not had biochemical mechanisms promoting resistance/tolerance to low ambient temperatures. In a number of studies [3–6], it was shown that, in contrast to homeothermic animals, their heterothermic counterparts with their flexible energy potentials demonstrate

throughout their evolutionary history the pivotal survival strategy which is determinative for life prolongation. While noting the adaptive superiority of heterothermic over homeothermic animals in responding to unpredictable environmental conditions, the authors emphasize that hypobiosis may give heterothermic species the advantage of being particularly adaptable to survival in permanently changing habitats.

Hibernation, genetically fixed during evolution, is an energy-conserving state of considerably reduced body temperature which allows animals of different phylogenetic levels and ecological niches to survive in adverse environmental conditions through minimization of their vital functions. While oxygen uptake and metabolic processes decline, the heart rate slows down at an extremely low body temperature, fluctuating in different species from +2 to +4°C [7] but sometimes dropping to –2°C [8]. Thus, in the winter period the European ground squirrel falls into a torpor at a certain periodicity, when body temperature reverts to its euthermic level for a few hours [9]. According to some authors [10, 11], hibernating species living in the arctic and subarctic regions show a very low metabolic level during hibernation at a temperature close to 0°C (chipmunk) and about (–1)–(–1.2)°C (long-tailed ground squirrel). A study by Solomonov et al. [12] demonstrated that the long-tailed (*Spermophilus undulates*, Pallas, 1778) and Arctic (*Spermophilus parryii* Richardson, 1827) ground squirrels in the torpid state have a body temperature below 0°C. Other authors [13], when analyzing molecular regulatory mechanisms in small mammals during prolonged hibernation, detected a drop in the metabolic rate more than by 90% and a decrease in the rectal temperature to 0°C.

In Arctic ground squirrels during hibernation there was found a 3- to 4-fold increase in blood and cerebrospinal fluid levels of one of the low-molecular antioxidants, ascorbate, by the end of torpor. Besides, there was revealed a decrease in enzymatic activity in tissues of animals at an extremely low body temperature of –2°C [7, 8]. In spite of vast studies of hibernating animals, the basic mechanisms of hypometabolism still remain a subject of debate. In experiments on insects there was supported the hypothesis that evolution of cold tolerance strategies in these invertebrates in extremely cold regions led to the production of highly specialized molecules that efficiently protect cells from lethal freezing [15]. Frost resistance of *Aporia crataegi* is based on the production of glycerol and ice-nucleating proteins. In the winter period, the *A. crataegi* caterpillar extract, having cryoprotective properties, contains the following amino acids: proline, asparaginic acid, serine, threonine and glutamic acid. There are studies by Karanova [16, 17] on the role of free amino acids in acclimation of the freshwater mollusk *Limnaea stagnalis* to near-zero temperatures and on the effect of cold shock on the free amino acid pool in the freshwater Amur sleeper *Perccottus glenii*. In some papers [18, 19], there were reported data on the variability of free amino acids in tissues of the leeches *Hirudo verbena carena* and *Hirudo medicinalis* both in different seasons and during adaptation to low positive temperatures in different climatic and geographical zones. Amino acid demand in heterothermic animals increases sharply during torpor because metabolic decline entails impairments in the protein synthesis both at initiation and elongation levels.

In the recent years, there has been a growing interest in bats as an experimental object, and vast information is accumulated on the number, species spectrum and habitation of chiropterans as well as on their bioenergetics at seasonal and temporary hypobiosis [20–23]. A research team [24] reported their findings on eco-physiological adaptation of five South American bat species in the torpid state. They found a high activity of antioxidant enzymes in their blood and tissues: α-tocopherol and β-carotene levels in the liver, kidney and cardiac as well as pectoral muscles were two orders of magnitude higher than in the liver of the tested rats and mice. Blood levels of reduced glutathione (GSH) and superoxide dismutase (SOD) as well as catalase activities were higher during hibernation than in active animals. The authors suggested that bats have an efficient antioxidant defense system capable of daily modulation to eliminate oxidative damages inflicted by hypoxia–reoxygenation when exiting the hypobiotic state. Antonova et al. [23] demonstrated the role of the antioxidant enzyme system in adaptation of hibernating chiropterans. The authors pointed out that a decrease

in catalase activity and an increase in that of superoxide dismutase in bat tissues represent one of the physiological mechanisms behind adaptation to periodical hypoxia–reoxygenation during hibernation.

Nevertheless, bats that developed during their evolution a high ecological plasticity and efficient cold tolerance strategies in ecosystems throughout whole continents remain a least studied group of heterothermic animals with regard to the formation of homeostatic mechanisms under conditions of prolonged near-zero thermal fluctuations in their habitats.

Chiropteran endemics of palearctic sylvatic biocenoses (*Mammalia, Chiroptera, Vespertilionidae*) have an important biological feature—the capability both of periodical inactivity during the day and prolonged hibernation during the winter season (5–7 months). Zoologists found out [20] that a most numerous boreal chiropteran species in the Ural region is the pond bat *Myotis dasycneme* which hibernates deep in the caves at a temperature from 0 to +2°C under conditions of extremely high humidity (90–100%).

The action mechanisms of many biologically active substances and biogenic amines providing mobilization of inner reserves of heterothermic organisms under low temperatures received rapt attention of researchers. Since free amino acids and their derivatives, being involved in the synthesis of structural proteins and serving as precursors of biogenic amines and some biologically active substances, are also considered in terms of their modifying effect on key metabolic processes [25] while the involvement of multifunctional free amino acids in metabolic provision of the adaptive response to negative and low positive temperatures in animals still remains debatable, our goal was to explore the free amino acid spectrum in chropteran blood plasma under experimental exposure of animals to low positive and near-zero temperatures.

Information available in the literature on metabolism of free amino acids in cryotolerant and cold-resistant representatives of bats (*Chiroptera, Vespertilionidae*) under exposure to low positive and near-zero temperatures during pre-hibernation is not presented in this paper.

Bats naturally adapted to the effect of low temperatures during hibernation serve a convenient model to study life reactivation after low-temperature stress they are exposed to in the nature. This paper addresses the problems of hibernation, specifically, the free amino acid spectrum in the bat blood plasma. It should be emphasized that in the chiropteran family *Vespertilionidae* inhabiting the Ural region this aspect of hibernation is studied for the first time.

MATERIALS AND METHODS

The pond bat *Myotis dasycneme* (n = 24) was caught by a mist net in the area of their mass habitation and winter/summer colonies in the Middle Ural near the Smolinsky cave (N 56°28', E 61°37') in the third decade of September 2015, i.e. in the course of their preparation for the winter season. During capture, the daily average ambient temperature in that territory was +10–12°C while in the cave it was from 0 to +2°C. In the laboratory experiment, only those bats were involved that showed no signs of diseases. To standardize experimental conditions, all animals were kept all the day through in a refrigerated chamber at +10–12°C. It is noteworthy that each bat chose its individual place in the container and showed no motor activity since then. Animals were in the resting state. Experimental group of bats was exposed to hypothermia through individual placement of animals into a refrigerated chamber at a temperature adequate to the ambient temperature in the cave (0–2°C) for 6 h. Capture and laboratory maintenance of animals complied with the regulations accepted at the European Convention (1986) on the protection of animals used for experimental and research purposes [26].

Physiological status of animals was assessed by the body temperature (as measured rectally by the TPEM-1 electrothermometer sensor) and the basal metabolism parameters [as recorded by oxygen uptake (mL/g × h) using the MN-5130 gas analyzer (Russia)]. Body mass was determined by weighing on the Acculab PP-200dl1 electronic balance at an accuracy of ±0.1 g. Blood was sampled after decapitation into sterile BD Vacutainer tubes with EDTA (UK).

Plasma levels of glucose and triglycerides were assayed by standard methods described in manu-

facturer's instructions. To obtain plasma, the blood was centrifuged at 3 000 rev/min and 0°C for 15 min in the K-23 D refrigerated ultracentrifuge (Germany). Plasma levels of glucose and triglycerides were assayed by the enzymatic colorimetric method using BioSystems kits (Spain). Optical density of the standard and experimental samples was measured on the SF-50 spectrophotometer (LOMO Spectr, Russia) at 500 nm. Plasma glucose concentration (detection limit: 0.0126 mmol/L) was calculated by the formula: $(A_{sample}/A_{stand}) \times C_{stand}$ (5.55 mol/L) = C_{sample}. Plasma triglyceride concentration (detection limit: 0.018 mmol/L) was calculated by the formula: $(A_{sample}/A_{stand}) \times C_{stand}$ (2.26 mol/L) = C_{sample}.

Plasma amino acid and urea assays. Samples were prepared for plasma amino acid and urea analyses according to a standard method [27]. The blood was centrifuged at 8 000 rev/min and 0—for 15 min in the K-23 D refrigerated ultracentrifuge. Upon cessation of centrifugation, plasma was aspired into a polyethylene tube where it was deproteinated. According to the standard recipe, 0.1 mL of 30% sulfosalicylic acid (SSA) was added to 0.5 mL of supernatant (plasma). 0.2 mL of 7% lithium hydroxide (LiOH) was added to neutralize the acidic reaction of the solution and 0.1 mL of norleucine ($C_6H_{13}NO_2$, 2.5 μmol/L; BIOLA-CHEMA-TEST, Czech Republic) as an internal standard. The tube content was repeatedly centrifuged at 10 000 rev/min and 0—for 30 min. Supernatant (400 μL) prepared for the analysis was applied onto a column.

Plasma levels of urea and amino acids were assayed by ion-exchange high-performance liquid chromatography (HPLC) on the AAA-339M automated analyzer (Mikrotechna, Czech Republic) at 38, 57 and 64—in the lithium–citrate buffer system containing a five-step gradient: no. 1—0.18 *n*, pH 2.90, no. 2—0.20 *n*, pH 3.1, no. 3—0.35 *n*, pH 3.35, no. 4—0.33 *n*, pH 4.05, no. 5—1.2 *n*, pH 4.9. HPLC column: 0.47 × 24.0 cm; stationary phase: Ostion LG FA cation exchange resin. Post-column amino acid modification was carried out with ninhydrin; its staining intensity was measured at 525 nm. For each experimental series, a chromatogram was recorded for the standard amino acid mixture comprising 36 components (0.1 μmol/L): cysteic acid, taurine, phosphoethanolamine, urea (10×), asparaginic acid, hydroxyproline, threonine, serine, asparagine, glutamic acid, glutamine, α-aminoadipinic acid, proline, glycine, alanine, citrulline, α-aminobutyric acid, valine, 1/2 cystine, methionine, cystathionine, isoleucine, leucine, tyrosine, phenylalanine, β-alanine, β-aminoisobutyric acid, γ-aminobutyric acid, ethanolamine, ammonia, ornithine, lysine, histidine, 1-methylhistidine, 3-methylhistidine, arginine (LaChema, Czech Republic). Norleucine (2.5 μmol/L; LaChema, Czech Republic) was used as an internal standard. For quantification, the amino acid chromaticity coefficient was calculated as a ratio of the peak square of an individual amino acid to that of norleucine. Plasma amino acid concentrations (μmol/L) were determined by a ratio of the obtained chromaticity coefficients for a sample and a standard mixture. For each tested sample, a whole spectrum of free amino acids was recorded on the chromatogram, with the concentration of each of them expressed both in μmol/L and as a percentage of the total amount. Cumulative concentrations were calculated for the following amino acids (AA): nonessential (NEAA), essential (EAA), glycogenic (GGAA), sulphurous (SAA), branched-chain (BCAA: valine+leucine+isoleucine), and aromatic (AAA: phenylalanine+tyrosine).

Statistical data analysis was carried out using StatSoft Statistica v. 10.0 and the R statistical computing environment. When testing differences in sample means between groups, $\bar{X}_{boot}$ [95% CI_{boot}], an arithmetic mean and bootstrap confidence interval, was determined [28]. When estimating the resultant differences between the tested groups, the Tukey's HSD test and Fisher's F test (ANOVA) were used. Nonparametric multivariate analysis of variance (NPMANOVA) and principal component analysis (PCA) were carried out using the R statistical computing environment (R 3.1.2; Ade4 package) [29].

RESULTS AND DISCUSSION

Against the backdrop of no differences in the body weight between animals involved in the experiment (Table 1), it was shown that in insectivorous chiropterans homeostasis of the internal environment is maintained due to an evolution-

Table 1. Morphophysiological parameters of the *Myotis dasycneme* and biochemical parameters of blood plasma during experimental cooling (0–2°C)

$\bar{X}_{boot}$ [95% CI_{boot}]				Variation source $p = Pr(\|F_{ran}\| \geq F_{obs})$	Permutation Two-way ANOVA ($p = Pr(\|F_{ran}\| \geq F_{obs})$
I. Control		II. Cooling			
1. ♂ ($n = 6$)	2. ♀ ($n = 6$)	3. ♂ ($n = 5$)	4. ♀ ($n = 5$)	*Exper* (1) Sex (2) 1×2	Tukey's Test ($p < 0.05$)
Body weight, g				0.61 0.63 0.43	$Pr(\|F_{ran}\| \geq F_{obs}) = 0.61$
16.8 [15.5–18]		17.3 [16.3–18.3]			
pO2, mL/g × h				0.14 0.0001 0.0001	1–3 2–4 3–4
5.1 [4.2–6.1]	6.1 [4.8–7.2]	12.2* [10.1–13.9]	1.2*@ [0.7–1.9]		
Rectal temperature, *t* °C				0.001 0.25 0.47	$Pr(\|F_{ran}\| \geq 19.05) = 0.001$
34.2 [32.4–35.3]		29.4* [27.9–30.6]			
1. ♂ (n=4)	2. ♀ (n=4)	3. ♂ (n=6)	4. ♀ (n=7)		
Triglycerides, mmol/L				0.0001 0.2 0.05	1–3 2–4
0.33 [0.22–0.44]	0.23 [0.16–0.3]	0.62* [0.55–0.69]	0.86* [0.68–1.03]		
1. ♂ (n=5)	2. ♀ (n=5)	3. ♂ (n=7)	4. ♀ (n=7)		
Glucose, mmol/L				0.46 0.21 0.04	2–4 3–4
4.51 [3.69–5.36]	3.71 [2.67–4.83]	4.6 [3.36–6.01]	2.77*@ [2.32–3.28]		
Urea, μmol/L				0.01 0.41 0.57	$Pr(\|F_{ran}\| \geq 12.32) = 0.01$
672.1 [411.4–1017.9]		1897.5* [411.4–1017.9]			

*—Statistically significant differences: *Control* and *Cooling*; *1* and *3*, *2* and *4* ($p < 0.05$); @—sex differences ($p < 0.05$); $\bar{X}_{boot}$ [95% CI_{boot}]—mean arithmetical and bootstrap confidence interval; $p = Pr(|F_{ran}| \geq F_{obs})$ —permutation (randomization) two-way ANOVA; *Exper*—*Experiment* factor.

ary consolidated strategy of behavioral and physiological adaptations. In bats, during the fall period of preparing for hibernation, after 6 h of experimental cooling (0–2°C) the rectal temperature in males and females dropped by 4.8—($p = 0.001$) but still remained within the physiological range of body temperatures in summer, which is 28.8 ± 1.7—[25.3–32.1] in males and 32.2 ± 0.3—[31.7–33.0] in females at a rate of oxygen uptake equal to 3.9 ± 0.41—[3.1–4.7] mL/g—h [30]. Basal metabolism in torpid males increased 2.4 times ($p = 0.0001$) (Table 1). There was detected a stable and quite high plasma glucose level in males. The glycemic level in females was by far lower than in males ($p = 0.04$). A drop in basal metabolism in females ($p = 0.0001$) exposed to near-zero temperatures was accompanied by a tendency to decrease the plasma glucose level. The plasma urea level in experimental animals was increased ($p = 0.01$). The plasma triglyceride level was increased 3.7 times in females ($p = 0.05$) and 1.9 times in males ($p = 0.05$). The significance of triglycerides as a main source of energy for normal functioning under low temperatures was also reported by other authors [31] (Table 1).

The plasma amino acid spectrum in tested *M.*

Table 2. Blood plasma amino acid content in the pond bat during experimental cooling (0–2°C)

Amino acids, μmol/L	$\bar{X}_{boot}$ [95% CI_{boot}]				Permutation Two-way ANOVA ($p = Pr(\|F_{ran}\| \geq F_{obs})$ Tukey's Test ($p < 0.05$)
	I. Control ($n = 9$)		II. Cooling ($n = 8$)		
	1. ♂ ($n = 5$)	2. ♀ ($n = 4$)	3. ♂ ($n = 4$)	4. ♀ ($n = 4$)	
Cysteic acid	6.5 [3.5–9.5]	14.2 [11–17.4]	47.9 [27.9–65.1]*	17.6 [10.5–27.1]@	1–3; 3–4
Glutamic acid	131.2 [105–159.6]		218.3 [180.7–257.1]*		$Pr(\|F_{ran}\| \geq 11.49) = 0.01$
Glycine	95 [82.7–110.3]		147 [116.5–179.4]*		$Pr(\|F_{ran}\| \geq 8.82) = 0.01$
Valine	25.6 [19.1–31.2]		80.6 [58.8–101]*		$Pr(\|F_{ran}\| \geq 20.75) = 0.001$
Methionine	7.7 [4.8–11]		19.7 [15.1–25.9]*		$Pr(\|F_{ran}\| \geq 13.89) = 0.001$
Isoleucine	14.5 [9–20.7]	11.3 [9–12.8]	6.4 [4.3–9.7]	21.8 [9.9–39.1]@	3–4
Leucine	25 [19.1–31.6]	16.6 [11.9–21.2]	23.2 [18.6–29.5]	43.4 [22.9–65.1]*	2–4
Tyrosine	13.3 [11–15.6]	13.8 [8.7–17]	14.8 [10.6–20.3]	38.1 [26–50.2]*@	2–4; 3–4
Phenylalanine	11.5 [9.3–13.7]		24.9 [18.1–32.8]*		$Pr(\|F_{ran}\| \geq 11.62) = 0.002$
Lysine	31.7 [26.4–37.9]	32.9 [27.4–38.3]	46.8 [40.1–53.6]*	81.7 [59.6–104.1]*@	1–3; 2–4; 3–4
Histidine	10.2 [7.8–12.3]		23 [18.2–28]*		$Pr(\|F_{ran}\| \geq 21.78) = 0.001$
Arginine	22.9 [19–27.4]	15.4 [6.1–32.6]	23.8 [12–36.9]	69.7 [54.2–90.9]*@	2–4; 3–4
Taurine	135.9 [82–210.1]		218.6 [145.6–304.8]		N. S.
Aspartic acid	39 [29.7–48.7]		52.9 [40.9–65.6]		N. S.
Threonine	75.7 [63.2–82.9]		78.2 [62.7–93.4]		N. S.
Serine	71 [57–86.4]		87.6 [67.3–107.7]		N. S.
Glutamine	78 [57.8–98.7]		62.8 [43.1–81.4]		N. S.
Alanine	265.8 [226.4–303.9]		274.5 [230.8–320.3]		N. S.
Cysteine	4.4 [1.4–7.9]		2.0 [0.4–3.7]		N. S.
Ornithine	40.5 [32.6–49.4]		53.3 [44.4–64.1]		N. S.
Asparagine	2.1 [1.7–2.6]	traces	traces	traces	–
Amino acid pool	1099.3 [960.5–1244.8]		1561.4 [1340.5–1782.3]*		$Pr(\|F_{ran}\| \geq 11.3) = 0.01$

N. S.—no statistically significant differences; other designations as in Table 1.

dasycneme individuals is presented by 21 amino acids (Table 2). NPMANOVA showed that bat males and females are statistically indistinguishable in total free amino acid concentrations both in control and experimental (cooled down to 0–2°C for 6 h) groups.

The plasma pool of free amino acids acts as one of the factors that are involved in low-temperature adaptation in bats during their preparations for hibernation: at 0–2°C, it increased (regardless of sex) by 42% and came to 1561.4 ± 112.6 μmol/L ($p = 0.01$) (Table 2). Such an appreciable increment during acclimation to low temperatures indicates a development of metabolic imbalance

caused by modification of nonessential and essential amino acids as predictors of incipient cryoprotective functions when animals prepare for a prolonged period of low temperatures which requires energy conservation [16–19, 32].

Analysis of plasma levels of essential and nonessential amino acids in control (pre-hibernating in fall) and experimental bats revealed no statistically significant differences in concentrations of taurine, aspartate, threonine, serine, alanine, glutamine, cysteine and ornithine ($p > 0.05$) (Table 2). It should be emphasized that the cumulative plasma level of alanine, glutamate and taurine is dominant both in the fall period of preparing for hypobiosis and in the experimentally induced torpid state. These specific amino acids accounted for 48% of the total pool of free amino acids in fall and 46% in the state of hypobiosis. A stable and high buildup of these amino acids suggests their leading role in the processes that ensure the survival of chiropterans at low temperatures, as additionally supported by a high plasma pool of glycogenic amino acids (34%) ($p = 0.01$) under cooling to 0–2°C. In males, the plasma level of the dicarboxylic amino acid asparagine decreases under cooling (0–2°C) to trace amounts whereas in females asparagine was detected in trace amounts only (Table 2).

The effect of tryptophan disappearance in the plasma of control bats was also observed at low temperatures (0–2°C). According to our previous findings, tryptophan is present in *M. dasycneme* tissues in the summer period of reproduction of the population [32]. It was found that the tryptophan level is significantly (3 times) higher in the liver of underyearlings than in adult individuals ($p < 0.05$). The hypothermic effect of the biogenic amine serotonin (or its precursor 5-oxytryptophan) was reported in ground squirrels [33] and hibernating golden hamsters [34]. Apparently, the lack of the essential amino acid tryptophan is a manifestation of one of the mechanisms of low-temperature adaptation: tryptophan seems to be spent completely for the synthesis of serotonin, one of the triggers involved in the maintenance of hypothermia and hypometabolism during the fall preparative period for hibernation [1].

It was shown that during cooling to 0–2°C blood plasma intensively accumulates essential amino acids, levels of which increase for glycine—1.5 times ($p = 0.01$), valine—3 times ($p = 0.001$), methionine—2.5 times ($p = 0.001$) (Table 2). Two aromatic amino acids begin to be actively utilized: phenylalanine by 216% ($p = 0.002$) both in males and females and tyrosine by 276% ($p = 0.01$) in females only (both are precursors of adrenaline and noradrenaline, catecholamines that play an important role in the mechanisms of hibernation in heterothermic animals) (Table 2). At 0–2°C, blood plasma intensifies accumulation of low-hydrophobic amino acids involved in the regulation and stimulation of physiological functions: lysine—1.5 times in males and 2.5 times in females ($p = 0.02$), arginine—4.5 times in females ($p = 0.003$), and histidine—2.3 times both in females and males ($p = 0.001$). This finding is supported by other studies of physiological strategies in cold resistant organisms which demonstrated the implication of these amino acids in protecting cell membranes from hypothermia-induced structural degradation [16, 17, 35]. Since prolonged hypothermia elicits a rise in the concentration of lysine, histidine and arginine, the suggestion about their cryoprotective action under low positive and near-zero temperatures is quite reasonable.

Blood plasma NPMANOVA allowed identification and quantitative estimation of metabolic groups of free amino acids that modify major metabolic pathways in the bat organism during torpor (gluconeogenesis, lipogenesis, tricarboxylic acid cycle) (Table 3).

Based on the results presented herein and the literature data analysis, we have demonstrated for the first time that during pre-hibernation of chiropterans protection of cell membranes from hypothermia-induced structural degradation is implemented due to amino acids with polar positively charged radicals (lysine, arginine) and histidine, which jointly ensure the survival of bats during the prolonged winter period (5–7 months) under exposure to low positive and near-zero temperatures. At the same time, tryptophan (essential amino acid serotonine precursor) deprivation promotes the development of adaptation of organisms to the long-term impact of low temperatures.

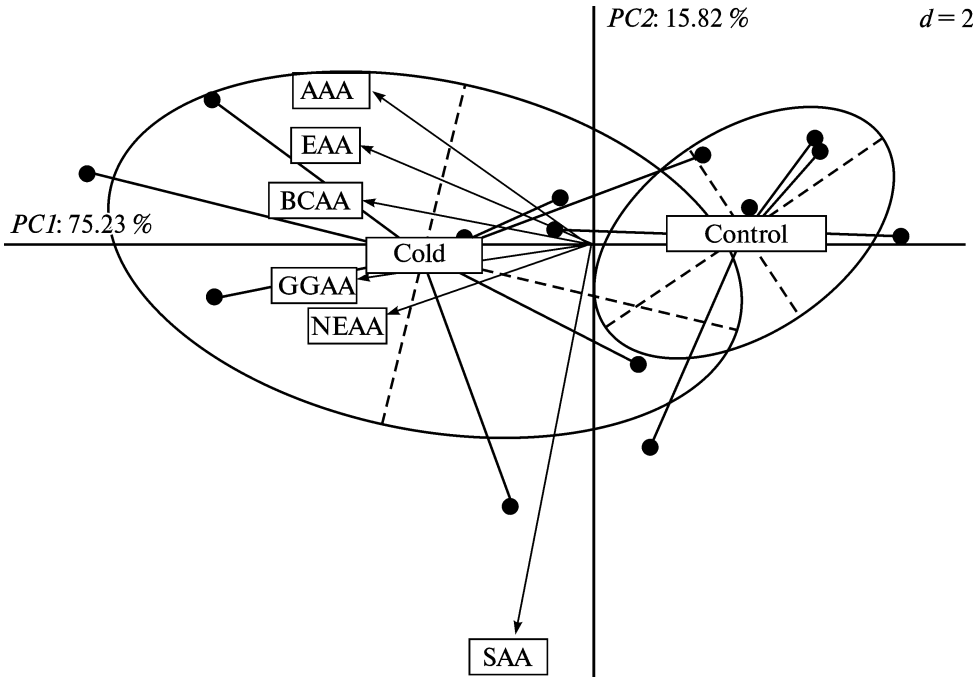
NPMANOVA (Ade4-PCA), used for the identification of inter-group differences in the amino acid spectrum and main metabolic amino acid

Table 3. Metabolic groups of plasma amino acids in the pond bat during experimental cooling (0–2°C)

Amino acids, μmol/L	Variation source $p=Pr(\|F_{ran}\| \geq F_{obs})$	$\bar{X}_{boot}$ [95% CI_{boot}]				Permutation ANOVA ($p = Pr(\|F_{ran}\| \geq F_{obs})$) Tukey's Test ($p < 0.05$)
		I. Control ($n = 9$)		II. Cooling ($n = 8$)		
	Exper (1)					
	Sex (2)	1. ♂ ($n = 5$)	2. ♀ ($n = 4$)	3. ♂ ($n = 4$)	4. ♀ ($n = 4$)	
	1×2					
Glycogenic (GGAA)	0.01	821.5 [727.2–915.4]		1092.2 [938.6–1261.5]*		$Pr(\|F_{ran}\| \geq 8.06) = 0.01$
	0.08					
	0.68					
Nonessential (NEAA)	0.04	699.3 [618–779.8]		872.3 [749.7–1012]*		$Pr(\|F_{ran}\| \geq 4.91) = 0.04$
	0.06					
	0.75					
Essential (EAA)	0.001	213.7 [191.3–237.1]		384.8 [316.2–462.1]*		$Pr(\|F_{ran}\| \geq 25.59) = 0.001$
	0.1					
	0.06					
Sulphurous (SAA)	0.07	157.8 [101.5–233.9]		272.4 [187.9–371.8]		N. S.
	0.96					
	0.11					
Branch-chain (BCAA)	0.001	59.9 [47.1–73]		127.8 [103.6–151]*		$Pr(\|F_{ran}\| \geq 23.47) = 0.001$
	0.49					
	0.15					
Aromatic (AAA)	0.0003	26.6 [24–30.7]	22.9 [19.9–25.7]	35.7* [30.5–44]	66.9*@ [48.5–84.1]	1–3
	0.04					2–4
	0.01					3–4

All designations as in Tables 1 and 2.

groups in *M. dasycneme* (control vs. cooling to 0–2°C) at a 95% confidence interval, shows that the first principal component (PC1) accounts for 72.2% of general dispersion of metabolic groups in the chiropteran plasma amino acid pool while the second principal component (PC2) accounts for 15.8% (figure, Table 4). By the presented variables, PC1 and PC2 determine statistically significant differences among bats in major metabolic groups of amino acids in the blood plasma (Table 4). Alteration of the plasma free amino acid pool after 6-h exposure to 0–2°C was significantly different from the control animals in the fall period. By the PC1, a major contribution to the variability of the amino acid pool is made by the following metabolic groups of amino acids: glycogenic, essential, branched-chain, nonessential, and aromatic (Table 4). By these variables and in accordance with the PC1, the animals were marked out in a separate group. It is necessary to emphasize a particularly high specific level of essential amino acids (20.7%) and their strong correlation with the PC1 (–0.97) (Table 4). We also demonstrated a high correlation coefficient of glycogenic amino acids with the PC1 (–0.95) and a high percentage of sulphurous amino acids (methionine and cysteine) as well as their strong correlation with the PC2 (–0.9) (Table 4). NPMANOVA allowed visualization of significant differences in the plasma amino acid spectrum of *M. dasycneme* (control vs. cooling), thus supporting the outcomes of the above-presented statistical analysis of free amino acids.



Metabolic groups of plasma amino acids (μmol/L) in pond bats *Myotis dasycneme* during experimental cooling (0–2°C) in the space of principal components PC1 and PC2. *PC1* and *PC2*—axes of two principal components; *%*—percentage of data variance explained by the principal component; *arrows* indicate correlation of principal components with initial values (metabolic groups of amino acids); *ellipses* represent 95% confidence regions.

Table 4. Component analysis of metabolic groups of plasma amino acids in the pond bat. Correlation coefficients for metabolic groups of plasma amino acids and principal components PC1 and PC2 (Ade4, R statistical environment)

Amino acid groups, μmol/L ($i = 6$)	Loadings, a_{ij}			Contribution to principal component $(a^2_{ij}*100)/\lambda_j$, %		
	Principal components (PC), j = 1, 2, 3					
	1	2	3	1	2	3
GGAA	# –0.95***	– 0.07	0.29	# 20.06***	0.59	21.79
NEAA	# –0.92**	– 0.09	0.38	# 18.61**	0.92	37.19
EAA	# –0.97***	0.18	–0.13	# 20.7***	3.47	4.52
SAA	–0.38	# –0.9***	–0.18	3.24	# 86.11***	8.64
BCAA	# –0.94***	0.08	–0.23	# 19.5***	0.69	13.6
AAA	# –0.9**	0.28	–0.24	# 17.89**	8.23	14.27
	Eigenvalues, λ_j, PC			PC-explained variance (%)		
	4.51	0.95	0.39	75.23	15.82	6.57

*—$p < 0.05$, **—$p < 0.01$, ***—$p < 0.001$. #—Amino acids with above-average contribution to the components calculated as 1/number of variables. Other designations as in Table 3.

Thus, we show herein that in the bats *M. dasycneme,* inhabiting various ecosystems of the Ural region, adaptation strategies to the impact of low positive and near-zero temperatures is realized due to considerable accumulation of free and essential amino acids (glutamic acid, alanine, threonine, glycine, valine, methionine, phenylalanine, lysine, arginine, histidine) under conditions of

near-zero temperatures, suggesting their cryoprotective role in providing the survival of heterothermic animals during hibernation. Cold resistance and reactivation of vital processes in bats during and after prolonged exposure to low positive and near-zero temperatures is based on physiological and molecular mechanisms of mobilization of the organism's reserves and spare capacities. Our results allow evaluation of the mediating (linking) role of free amino acids in the integration of metabolic processes which had evolved in the long-term evolution of Chiroptera, making them highly resistant to the effect of near-zero environmental temperatures during prolonged hypobiosis. We also believe that our results allow taking a fresh look at the cryoprotective function of free amino acids in heterothermic animals, creating prerequisites for predicting post-hypothermic consequences in modeling and correcting hypobiotic conditions for medical and biotechnological purposes as well as for cryobiology. Chiropterans, naturally acclimated to the impact of low temperatures during hibernation, can serve as a handy model to explore life reactivation processes after low-temperature stress.

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