THE ECO-PHYSIOLOGICAL STATUS OF HIBERNATING BATS (CHIROPTERA) IN THE NORTH OF THE EUROPEAN DISTRIBUTION RANGE

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The chiropteran fauna in hibernacula in Karelia is chiefly made up of widespread sedentary boreal species – the northern bat *Eptesicus nilssonii (*Keyserling & Blasius, 1839), Brandt's bat *Myotis brandtii* (Eversmann, 1845) and brown long-eared bat *Plecotus auritus* (Linnaeus, 1758). The Daubenton's bat *M. daubentonii* (Kuhl, 1817) and especially the whiskered bat *M. mystacinus* (Kuhl, 1817) are far more rare, and wintering of the pond bat *M. dasycneme* (Boie, 1825) and the Natterer's bat *M. nattereri* (Kuhl, 1817) has not yet been confirmed. The comparative study of the immune status, antioxidant system and lactate dehydrogenase (LDH) isoenzyme spectra in tissues (liver, heart, kidney, lung, spleen and skeletal muscle) of three *Chiroptera* species during hibernation was conducted. It was revealed that torpor drastically reduces the numbers of all types of circulating leukocytes. In addition, changes have been noted in the antioxidant system, such as the relatively high antioxidant protection displayed by their tissues, considering both constitutive antioxidant enzymatic activities and nutritional antioxidants. The LDH isoenzyme spectra in bat kidneys and heart had the high contents of hybrid fractions. Superoxide dismutase and catalase activities, vitamin E levels and leukocyte differential count were significantly different in three bat species. The differences may be due to the species-specific biological and eco-physiological features.

Keywords: bats, hibernation, resistance, antioxidant system, LDH isoenzyme spectra, leukocytes.

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INTRODUCTION

Hibernation is a conservative strategy that animals adopt to conserve the energy needed to survive under demanding conditions. In the winter season, small mammals become torpid, drawing their body temperature down to approximate that of the environment, i.e. to around 10 °C as a rule, but drops to -2 °C are possible (Heldmaier et al. 2004). Depending on the animal species and the ambient temperature, a torpor bout may lasts four to fourty days, regularly interspersed by brief euthermic arousal phases (Heldmaier et al. 2004).

The choice of hibernacula by bats is known to be species-specific and to vary among regions. The main criteria for the choice of hibernation habitats are air temperature and humidity, which generate a certain microclimate in the shelters to secure that the animals do not freeze or dry out (Кузякин 1950, Калабухов 1985, Wermundsen & Siivonen 2010). Since such shelters are very scarce, many species have to migrate both towards wintering grounds and between several shelters, and the animals have to select proper sites within shelters (Мазинг 1990, Kokurewicz 2004). By choosing optimal settings for hibernation, bats can manage their energy consumption, reducing energy loss to a minimum (Kokurewicz 2004, Ануфриев & Ревин 2006). The weather and physiological stimuli arouse the animals from hibernation for various kinds of activity – movements, feeding, drinking, mating.

Hibernating bats exhibit appreciably lower demand for resources (Geiser & Ruf 1995, Boyer & Barnes 1999, Carey et al. 2003, Geiser 2004). Although metabolic rates during torpor are incredibly low, the expression of some genes and proteins is elevated to satisfy current needs of the organism (Eddy & Storey 2007). Thus, the energies of all organs during hibernation are based on lipid oxidation, as manifest in multiple adaptive metabolic shifts that intensify the catabolism of triacylglycerols while at the same time suppressing the catabolism of carbohydrates. The metabolic shifts the organism requires during hibernation involve activation of one of transcriptional targets of HIF-1, pyruvate dehydrogenase kinase isoenzyme 4 (PDK-4) (Buck et al. 2002), as demonstrated for the skeletal muscle of hibernating thirteen-lined ground squirrels. PDK-4 upregulation is probably promoted by the HIF-1 factor, which hinders normal carbohydrate metabolism by inactivating pyruvate dehydrogenase (Maistrovski et al. 2012).

Resistance mechanisms in the animals entering hibernation have so far been poorly studied. As the animals adapt to low temperatures a majority of physiological-biochemical parameters – breathing rate and heart rate, blood pressure, urea excretion rate, blood delivery to organs, oxygenation, haemotopoiesis, and others – undergo significant changes (Bouma et al. 2010, Hochachka & Somero 1984). Presumably, changes during hibernation are aimed to mitigate damage at repeated torpor/arousal transitions. Longer cold torpor bouts alternate with shorter arousal periods, during which the animal returns to euthermic state. These cycles are closely connected to substantial fluctuations in oxygenation, tissue perfusion and ATP cycle. Hence, arousal appears impossible without an oxidative stress associated with a colossal rise in oxygen consumption necessary to maintain brown adipose tissue and skeletal muscle thermogenesis (Allen & Storey 2012). An advanced antioxidant protection is essential under these circumstances, being an indispensable part of all types of stress-induced hypometabolism (Storey et al. 2007). The antioxidant system secures long-term vitality of the cell during hypometabolism periods, when the degradation and re-synthesis of oxidatively damaged macromolecules is minimal, as well as facilitates a ‘gentle’ transition of the organism from the hypometabolic state to normal activity.

Information about the composition and morphology of blood cells playing a key role in the adaptation and immunoreactivity of the organism in bats is also scant (Bouma et al. 2010). Quite possibly, it was changes in the immune status of hibernating bats that were

the cause of the white-nose syndrome (WNS) and massive mortalities of bats in the USA and Canada in 2012. Evidence was found that the deaths were due to nose and wings damaged by the fungus that grew optimally at 12.5-15.8 °C, but could develop quite well even at lower temperatures – 3-6 °C (Bouma, Carey, Kroese 2010).

Our studies have focused on the ecology and physiology of vesper bats in the winter season in the Republic of Karelia, where underground hibernacula were found below 63° N, as well as on identifying linkages between the species abundance and its physiological-biochemical parameters during hibernation.

MATERIAL AND METHODS

Field surveys were carried out in the middle taiga subzone in southern Karelia (61−63° N, 30−36° E). Six underground hibernacula spaced 70−300 km apart were surveyed (Fig. 1). They have not previously been studied or mentioned in specialized literature. Using the classification by M. Mazing (Мазинг 1990), they can be described as man-made caves (№ 2, 3, 4, 6) and lined crypts (№ 1, 5). The man-made caves are former iron, marble and granite mines, and the crypts are former bomb shelters cut out in rock.

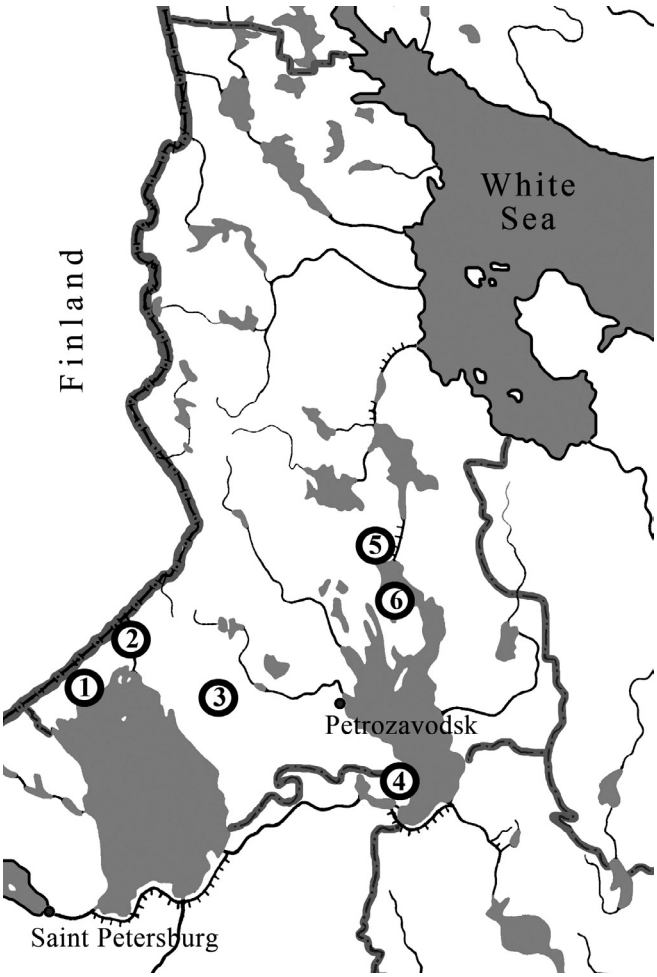

Fig. 1. Locations of bat hibernacula in the Republic of Karelia. Legend: 1 – Lahdenpohja, 2 – Ruskeala, 3 – Sona, 4 – Shcheleyki, 5 – Medvezhjegorsk, 6 – Shunga.

The bat census at hibernacula and sampling of the biological material were carried out in October to April of 2009–2014 by inspecting caves and crypts throughout (Белкин et al. 2013). The easily differentiated species were identified visually (brown long-eared bat, northern bat), whereas myotids (Daubenton's, Brandt's and whiskered bats) were examined for the position of the wing membrane attachment to the hindlimb – the main trait for *in situ* differentiation between species in this chiropteran group. Differentiation between *M.brandtii* and *M. mystacinus* was performed only for males based on penis shape (Стрелков & Бунтова, 1982, Кожурина 1997, Schober & Grimmberger 1997). Because of the profound phenotypic similarity, the rest of the Myotis bats were grouped together as *M. brandtii/mystacinus,* as has been done also by Finnish zoologists (Siivonen & Wermundsen, 2008). Some specimens were diagnosed

within the analysis of craniological material. The total count was 149 bats, and if data from Lahdenpohja in 2002−2003 submitted by biologist V. Kusakina are taken into account the number is 153 bats. Selective sampling of bats from hibernacula was carried out with permits from the Game Management Directorate of the Republic of Karelia (№№ 0002-2010, 0001-2011, 00008-2013) and in compliance with the international guidelines for ethical treatment of animals laid out in the Helsinki Declaration (Этическая экспертиза… 2005).

The physiological-biochemical and morphological indices were determined using in total 7 northern bats, 5 Brandt's bats and 2 brown long-eared bats. Animals were collected from their natural habitats in February-March.

The samples of tissues were collected after decapitation, then snap-frozen and stored at –25 °C for further analysis. Different values were determined in the tissue samples. To assay LDH patterns and determine the activity of antioxidant enzymes tissue samples were homogenized in 0.05 M phosphate buffer, pH 7.0, and centrifuged at 6000g for 15 min. Preliminary tests revealed no differences between sexes, hence, data for males and females were pooled together in all subsequent analyses.

The total SOD activity was measured by the adrenochrome method based on spontaneous autoxidation of epinephrine with the formation of product with an absorbance peak at 480 nm (Misra & Fridovich 1972). This reaction depends on the presence of superoxide anions and is specifically inhibited by SOD. The amount of enzyme that caused 50% inhibition of epinephrine autoxidation was defined as 1 unit (U). SOD activity was expressed as U per g raw tissue weight. Catalase activity was evaluated by measuring the decrease in H_2O_2 concentration at 240 nm (Bears & Sizes 1952). One enzyme unit (IU) was defined as the amount of catalase capable of transforming 1.0 μmol of H_2O_2 for a min. Catalase activity was expressed as IU per g raw tissue weight. The tissue concentrations of vitamins A (retinol) and E (α-tocopherol) were determined by high performance liquid chromatography with ultraviolet detection at 324 and 292 nm, respectively (Skurihin & Dvinskaya 1989). This is an isocratic method using hexane:isopropanol (98.5:1.5) as the mobile phase. Separation of the LDH isoenzymes was performed by Wieme's method of horizontal electrophoresis on agar gel plates (Wieme 1959). The LDH isoenzyme ratios were estimated quantitatively after histochemical staining of samples by scanning electrophoregrams. The content of LDH isoenzymes was expressed as percentage of the total enzyme activity. Total white blood cells (WBC) count was performed using Goryaev chamber under light microscope (Axioscop 40, Zeiss). Differential leucocyte counts were done by counting 200 cells in blood smears stained by Pappenheim method with May-Grünwald and Romanowski stain (MiniMed, Russia). The absolute numbers of each type of leukocytes were calculated from the WBC and differential leukocyte count.

All the collected and calculated numerical data were processed statistically as mean ± standard error of the mean. Statistical analysis was performed using the nonparametric Mann-Whitney U-test or χ^2 test. Differences between samples were considered to be significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

The censuses of bats at hibernacula have shown that at least five species stayed there over winter: Brandt's bat (7.3% of the total number of counted animals), whiskered bat (0.7%), Daubenton's bat (4.6%), brown long-eared bat (5.3%), northern bat (72.8%). Some of the animals were conventionally pooled into the Brandt's/whiskered bats group (9.3%).

The composition and ratios of species differed significantly among hibernacula in Karelia, but an overall prevalence of the northern bat was found both in caves and in lined crypts. As opposed to other species, the northern bat was

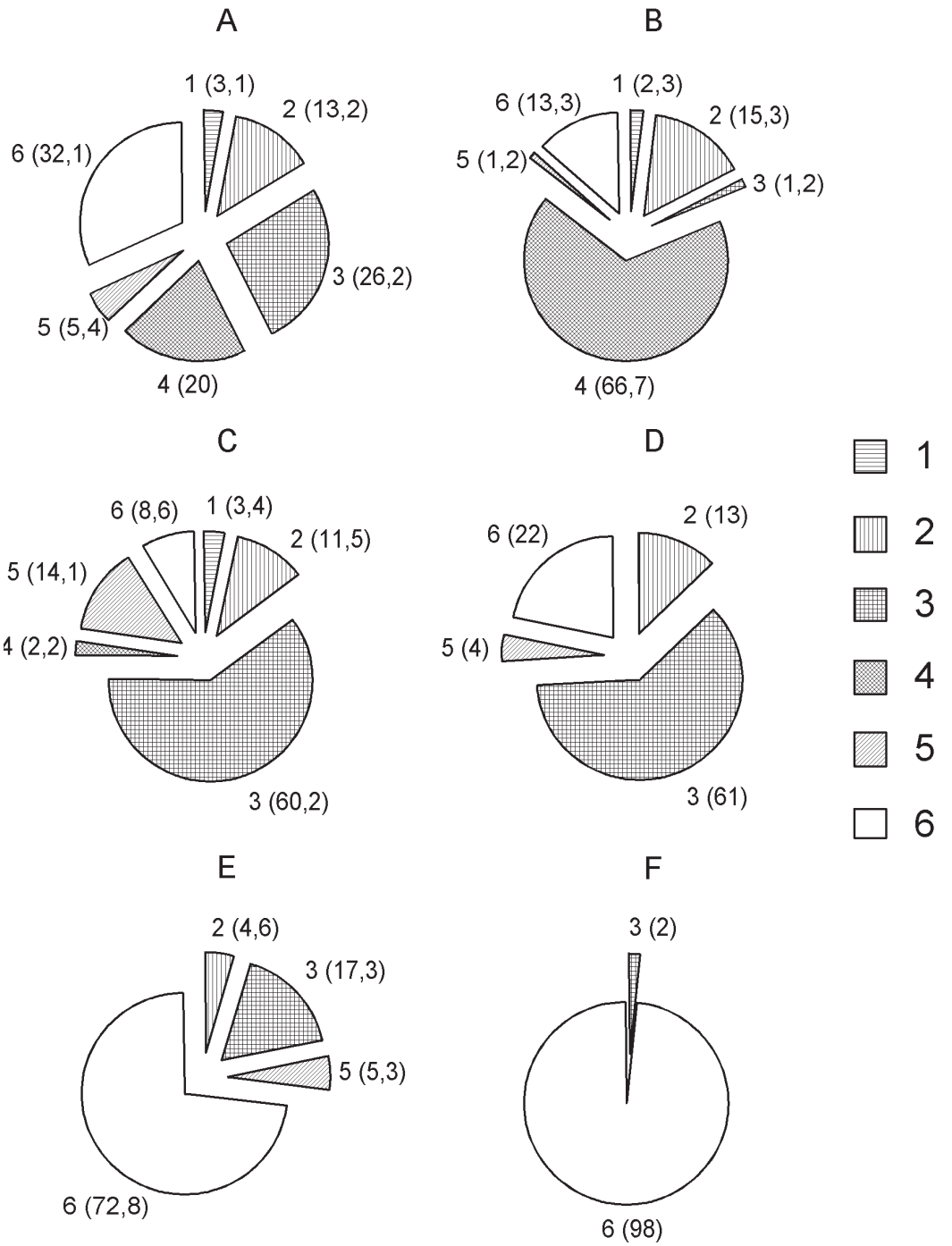

Fig. 2. Species structure of hibernating bat populations (% of the total number of animals in the counts): A – Leningrad Region (Стрелков 1958), B – Tver Region (Глушкова et al. 2006), C – Samara Region (Смирнов et al. 2012), D – Finland (Siivonen & Wermundsen 2008), E – Karelia (own data), F – Arkhangelsk Region (Рыков 2008). 1 – *M. dasycneme*, 2 – *M. daubentoni*, 3 – *M. brandtii*/*mystacinus*, 4 – *M. nattereri*, 5 – *P. auritus*, 6 – *E. nilssonii*.

present in all the surveyed hibernacula, with 1 to 20 specimens in each. The Brandt's/whiskered bats were spotted in three caves, but not every year (1 to 6 specimens), the brown long-eared bat – in three caves and one concrete-lined crypt not every year (1−2 sp.), the Daubenton's bat – in three caves, 1-3 specimens in each, not every year, and the whiskered bat was encountered only once.

The spatial distribution of bats within hibernacula of different kinds varied significantly. In lined crypts, 90.9% of the animals roosted on walls (a half of them – at the wall-ceiling junction, and the rest – higher than 1 m from the floor) and only 9.1% − on the ceiling (χ^2=66.9, p=0.01). In fact, 22.7% of the bats were hiding behind the concrete and brick lining rather than stayed in the halls (χ^2=29.89, p=0.01). The distribution of bats across caves was different: only two thirds of the animals occupied walls, usually not lower than at 1 m, while the rest stayed on the ceiling (χ^2=16.0, p=0.01), most of them openly, ignoring the numerous boreholes (χ^2=7.5, p=0.01). Pair-wise comparisons of the locations of hibernating bats within man-made caves in Karelia showed that myotids preferred walls to the ceiling (χ^2=65.9, p=0.01) and boreholes to open spaces (χ^2=14.1, p=0.01). Furthermore, Myotis bats often congregated in clumps of 2-4 animals (45.5% of specimens), whereas Northern bats stayed individually (χ^2=60.5, p=0.01) and openly (χ^2=26.2, p=0.01), chiefly on walls (χ^2=4.42, p=0.05).

Bat mortality at hibernacula was observed only in the biggest man-made caves – in Ruskeala and Sona. Of the 92 animals counted there 10 individuals were found dead (10.9%): 7 northern bats, 1 Brandt's bat and 2 Daubenton's bats. The mortality of bats was 6.5% of the total number of animals counted throughout the period of observations (*n*=153). Of the 110 northern bats in the sample 6.4% had died, of the 26 Myotis bats in the Brandt's/whiskered bats group 8.3% had died, of the 7 Daubenton's bats – 28.6%, and no dead brown long-eared bats were found among the 8 specimens in the count.

The bat species ratio determined for Karelia was notably different from that reported for SE Finland (Siivonen & Wermundsen 2008), northern Leningrad Region (Стрелков 1958) and Arkhangelsk Region (Рыков 2008). The differences consisted in the significant prevalence in the hibernacula below 61° N of various myotid species (relative abundances – 64.8 and 74.0% in the Leningrad Region and Finland, respectively), in the low share of hibernating brown long-eared bats in Karelia and Finland (4.9 and 4.0%, respectively) and their high share in the Leningrad Region (32.1%),

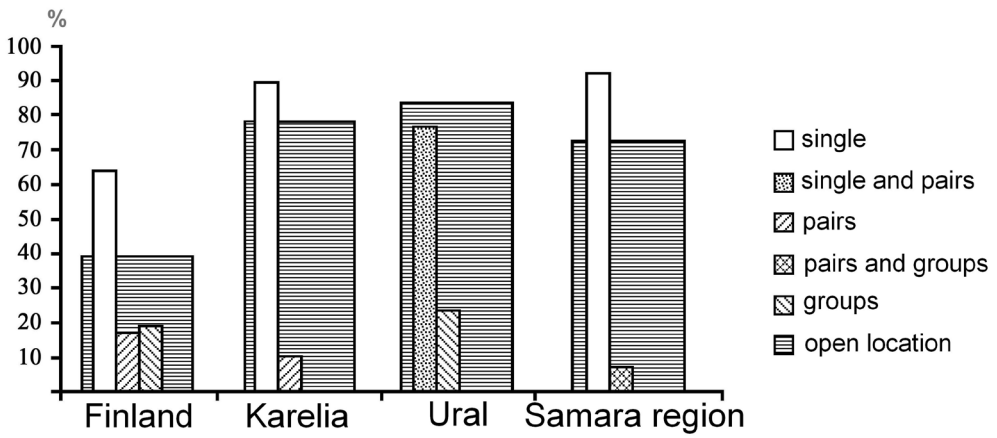

Fig. 3. Distribution patterns of the hibernating northern bat, % of the total number of animals in counts: Finland (Siivonen & Wermundsen 2008), Karelia (own data), Ural region (Орлов 2000, Большаков et al. 2005), Samara Region (Смирнов et al. 2008, Смирнов & Вехник 2009).

in an absolute dominance of the northern bat in Karelian hibernacula – 78.2% (Fig. 2). In the Arkhangelsk Region, near Pinezhsky Strict Nature Reserve (64°33′ N, 43°11′ E), the share of the northern bat in chiropteran communities was even higher – relative abundance over 98% (Рыков 2008).

Data on bat hibernacula in southerner parts of Russia and in Estonia (Fig. 2) generally fit in the overall pattern of latitudinal change of the bat population species structure: prevalence of four or five myotid species, low share of the northern bat below 61° N, and dominance of the latter higher than 61° N. Equally illustrative is the distribution of the brown long-eared bat in hibernacula: its abundance was the highest in the Leningrad Region (59−60°30′ N), decreasing southwards (Tver and Samara Regions, 53−56° N) and especially so north of the Leningrad Region (southern Finland and Karelia, 60°30′−63° N; Arkhangelsk Region, 64°33′ N).

On the other hand, some species-related features of bat hibernation in Karelia agree with data from the much southerner caves of the Ural region (Большаков et al. 2005) and underground hibernacula in the Samara meander of the Volga (Смирнов et al. 2008) (Fig. 3).

Winter seasons in Karelia feature frequent and abrupt weather shifts, considerable deviations of mean monthly December-March temperatures from the climatic norm (by 7.1°C at cooling and by 6.8°C at warming). According to Strelkov (Стрелков 1965), animals die late in the cold season (March-April), and our data corroborate this assumption. Data on bat mortality in other wintering areas are very scarce (Стрелков 1965, Мерзликин 2002, Ануфриев 2007), reporting only few occasional deaths.

Thus, the chiropteran fauna in Karelian hibernacula is primarily made up of wide-ranging boreal sedentary species – Brandt's bat, brown long-eared bat, and northern bat. The Daubenton's bat and especially the whiskered bat are far rarer, and habitation of the pond bat or the Natterer's bat has not yet been confirmed even for the summer season. All the above-mentioned species have been reported from southern Finland, and a single case of hibernation of the particoloured bat *Vespertilio murinus* (Linnaeus, 1758) has been recorded from the Leningrad Region, near the border with Karelia (Новиков et al. 1970, Siivonen & Sulkava 1999, Siivonen & Wermundsen 2003).

A comparison of our data with materials from central and southern Russia (Стрелков & Ильин 1990, Золина et al. 2007, Безруков & Каменек 2008, Ситникова et al. 2009, Чистяков 2009) showed that the species composition of bats grew poorer northwards, as well as eastwards (Ануфриев 2008), dropping to a minimum in the Palearctic boreal belt between 60 and 63° N. Yet, such a reduction in the number of chiropteran species along the latitudinal gradient is not so sharp as in other European countries (Pereswiet-Soltan 2007). It has been demonstrated (Ulrich et al. 2007) that the areas, geographic latitude and the annual range of air temperatures account for 73% of the total variation of the species richness of bats in 58 European countries. The representation of chiropteran families, genera and species declines from the Mediterranean to East European countries, with a minimum in Estonia and Finland.

These variations in the frequency of occurrence of different species may be related to their specific physiological-biochemical characteristics, in particular the status of the antioxidant and the immune systems. Metabolic shifts in hibernating animals first of all affect the heart, as the organism switches from carbohydrates to lipids as the main source of energy (Eddy & Storey 2004). Glycolysis results in lactate accumulation and acidification of the medium, causing a higher pressure on the kidneys. It was the kidneys and the heart that showed the greatest number of differences in the distribution of LDH isoenzymes in the studied species (Table 1). Although the heart is traditionally perceived as an organ where the first and the second anode fractions prevail (Echigoya et al. 2009), its LDH isoenzyme

Table 1. Lactate dehydrogenase isozymes distribution and content H and M subunits in the tissues of bats

Species	Tissue	LDH isoenzymes, %					Subunits, %	
		LDH-1 (HHHH)	LDH-2 (MHHH)	LDH-3 (MMHH)	LDH-4 (MMMH)	LDH-5 (MMMM)	H	M
P. auritus	heart	0	0	97.89	2.11	0	49.47	50.53
	kidney	0	0	12.87	36.98	50.15	15.68	84.32
	skeletal muscle	50.86±1.34	32.24±3.77	9.43±2.7	6.14±2.34	1.33±0.08	81.29	18.71
M. brandtii	heart	2.82±1.85	0.58±0.50	85±5.15	10.51±5.54	1.09±0.4	48.38	51.62
	kidney	0.47±0.34	0.09±0.09	30.73±3.71	24.97±3.99	43.73±5.53	22.15	77.85
	skeletal muscle	59.43±3.39	30.12±1.14	6.51±2.75	2.51±0.8	1.43±0.69	85.9	14.1
E. nilssonii	heart	2.29±1.91	0	77.76±8.29	18.31±7.43	1.63±0.33	45.75	54.25
	kidney	1.51±0.81	0.99±0.66	37.22±5.87	33.84±2.64	26.44±5.91	29.32	70.68
	skeletal muscle	59.43±2.75	31.72±1.5	6.75±1.92	1.88±0.57	0.23±0.15	87.06	12.94
Values are means ± s.e.m.								

spectrum in bats was found to contain high amounts of the LDH-3 isoenzyme (77 to 98%). We have previously demonstrated a similar distribution pattern for another hibernator – the northern birch mouse *Sicista betulina* (Pallas, 1779). The isoenzyme spectrum in its heart and kidneys was dominated by hybrid fractions (Антонова, Хижкин, Полетаева 2012). For those tissues that periodically experience both aerobic and anaerobic conditions simultaneous presence of both H- and M subunits of isoenzymes is the most beneficial, since a substantial part of LDH molecules will then be of the hybrid type (Кожевникова et al. 2004). While having nearly the same enzymatic activity, LDH isoenzymes differ in their affinity for substrates and cofactors. The change in the qualitative composition of subunits is possibly due to a reduction in enzymatic activity during torpor, and kinetic differences between subunits enable the organism to adapt to variations in the environment.

The prevalent fractions in the LDH isoenzyme spectrum in the kidneys of bats were LDH-3, LDH-4 and LDH-5, contradicting the classic distribution found in kidney tissues in most non-hibernating mammals (Кожевникова 1987, Echigoya et al. 2009, Daneshrad et al. 2003). The presumable reasons for that are reductions in the breathing rate, blood circulation rate and suppression of most metabolic processes during hibernation. As a result, organ perfusion is reduced to a value that under normal conditions would be interpreted as ischemic (Frerichs et al. 1994). Most likely, hypoperfusion of kidneys shifts the metabolism towards anaerobiosis and prevalence of M subunits. A rise in lactase concentration in the plasma found in bats during arousal periods (Lee, Choi, Park 2002) may be a consequence of its excretion from kidneys and other organs during intensification of circulation processes. A peculiar fact is that the northern bat had the lowest content of the LDH-5 fraction, and the highest content of the LDH-3 fraction. The pattern in the brown long-eared bat was the opposite. The high content of H subunits in kidneys of hibernating northern bats (29.3%), as compared with brown long-eared bats (15.7%), may be either species-specific or associated with the physiological features of the hypothermal period. Hibernation in the northern bat was found to occur at lower temperatures (2.0±0.1ºC) and relative humidity (78.0±0.6%)

as compared with other species – Brandt's bat, whiskered bat, Daubenton's bat, as well as brown long-eared bat (Siivonen & Wermundsen 2008).

A specific distribution of LDH isoenzymes was detected also in the skeletal muscles of the species in question. The total content of the first and second anode fractions ranged from 83.1% in the brown long-eared bat to 91.4% in the northern bat. In non-hibernating mammals, skeletal muscles are traditionally described as tissues where the fourth and fifth cathode fractions prevail. Presumably, skeletal muscles during hibernation feature high rates of aerobic oxidation of lipids, which provide for thermogenesis during periodic arousals (Storey & Storey 2004). Contractile thermogenesis requires high amounts of energy, whereas the glycolytic yield is low compared to oxidative phosphorylation. The high content of H subunits in the skeletal muscles of bats is probably an adaptation to gain more energy. The lowest content of M subunits in skeletal muscles was found in the northern bat (12.9%), the highest – in the brown long-eared bat (18.7%), and the Brandt's bat held an intermediate position (14.1%). The LDH isoenzyme distribution in the skeletal muscle in these animals could have been influenced by the duration of torpor bouts. Thus, as reported by Anufriyev (Ануфриев 2008), hypothermia lasted the longest (97.33% of the time) in northern bats. They also demonstrated the longest average duration of a torpor bout (428 hours). In contrast, these values in the brown long-eared bat were 96.37% and 225 hours, respectively. The Brandt's bat held an intermediate position.

In this study we found a species-specific and tissue-dependent distribution of antioxidant enzyme activities (Table 2). Thus, the highest activity levels were observed in the liver, and the lowest – in the muscles of all three species in the case of catalase, and in the kidneys of brown long-eared bats as well as in the lungs of Brandt's bats and northern bats in the case of SOD. Since the liver is central to intermediate metabolism, high antioxidant enzyme activities were apparently associated with this function of the organ. Species-specific patterns of AOE activities were distinguished only in the specific activities of enzymes. SOD activity in the skeletal muscle of the Brandt's bat was lower than in the northern bat. On the other hand, the Brandt's bat exhibited a higher catalase activity in the liver and a lower activity in kidneys compared to the northern bat.

The skeletal muscle is crucial for locomotion, including hunting, escape and reproduction. Atrophy may be a serious problem for skeletal muscles during hibernation. In all non-hibernating mammals, muscles would shrink after a prolonged absence of contractions (Storey & Storey 2012). In fact, there is little evidence of atrophy during hibernation (Tessier & Storey 2010), which may be a hint that hibernators have optimized the mechanisms for mitigating/preventing muscle wasting (Storey & Storey 2012). In clinical and experimental models, muscle resting in mammals, e.g. limb immobilization, has led to muscle wasting and reduced their contractile capacity (Musacchia et al. 1988, Powers et al. 2007, Clark 2009). During torpor bouts hibernators show no visible motions, although previous studies have demonstrated a relatively low degree of muscle dystrophy compared to artificial models of muscle resting (Musacchia et al. 1988, Hudson & Franklin 2002, Shavlakadze & Grounds 2006). Quite a number of mechanisms contribute to the resistance of hibernators to muscle atrophy, including enhanced expression of antioxidants (Hudson & Franklin 2002, Allan & Storey 2012), reduced levels of myostatin (Braulke et al. 2010, Brooks et al. 2011, Nowell et al. 2011), and regulation of the transcription factors of the genes associated with muscular activity (Tessier & Storey 2010). The total antioxidant capacity of the gastrocnemius was found to be 156% higher in torpid animals compared to summertime specimens (James 2013).

Elevated antioxidant, glutathione and SOD levels prevent the adverse oxidative effects of AOS, mitigate cell damage and potential muscle wasting (Carey et al. 2003, Powers et al. 2007).

Table 2. The specific activity of antioxidant enzymes and protein content in bat tissues

Species	Tissue	Indices, M±m		
		Activity of SOD, units/mg of protein	Activity of catalase, arb. units/mg of protein	Content of protein mg/g tissue
P. auritus	liver	3.56±0.55	2.87±1.02	191.83±30.04
	kidney	1.06 (n=1)	0.66 (n=1)	132.96 (n=1)
	heart	1.50 (n=1)	0.24 (n=1)	101.31 (n=1)
	lung	1.11±0.32	0.38±0.08	186.33±15.27
	spleen	0.89 (n=1)	0.71 (n=1)	369.57 (n=1)
	skeletal muscle	2.93±1.03	0.24 (n=1)	116.60±27.64
M. brandtii	liver	2.96±0.37	2.65±0.35	187.01±20.26
	kidney	3.48±1.40	0.48±0.05	132.99±6.26
	heart	1.93±0.29	0.26±0.01	121.34±7.27
	lung	0.64±0.17	0.34±0.03	175.22±9.29
	spleen	2.25 (n=1)	0.59 (n=1)	123.82 (n=1)
	skeletal muscle	2.14±0.22	0.25±0.04	118.95±7.90
E. nilssonii	liver	3.78±0.27	1.77±0.16*	211.99±6.17
	kidney	3.37±0.96	0.82±0.16*	108.24±10.68
	heart	2.31±0.27	0.30±0.03	117.69±5.13
	lung	0.59±0.10	0.33±0.04	184.52±8.04
	spleen	1.54±0.41	0.59±0.10	222.69±14.59
	skeletal muscle	3.55±0.41*	0.60±0.10	84.09±13.32

Values are means ± s.e.m. * - differences in the same tissue are significant in comparison with *M.brandtii* ($p<0.05$).

In hibernating 13-lined ground squirrels *Citellus tridecemlineatus* (Mitchill, 1821) however, most AOS are not upregulated in brain, liver and heart (Page et al. 2009). To secure an adequate level of antioxidant protection, bats, like other hibernators, show preference for low-molecular antioxidants (Drew et al. 2004, Wilhelm-Filho et al. 2007).

Vitamin E inhibits metabolism in tissues, and tocopherol level is an important regulator of the physiological condition of the animals that hibernate in winter. The northern bat had a significantly higher content of α-tocopherol in liver, kidneys and skeletal muscle than other bat species (Fig. 4). However, no considerable differences in the values between the northern bat and the brown long-eared bat were detected in the heart, indicating a high stability of antioxidant defense in this tissue in all bats. Vitamin A content in the liver was quite similar in the Brandt's bat and the brown long-eared bat, but the northern bat had the lowest retinol concentration in the liver. Other tissues contained low vitamin A levels or the retinol concentration was not detected.

Vitamin E is the most important natural antioxidant and its sufficient uptake is important, especially in northern regions, before the winter period in connection with significant metabolic changes. The uptake, transport and tissue delivery of α-tocopherol, a key vitamin E form, involved molecular, biochemical, and cellular processes closely related to overall lipid and lipoprotein homeostasis (Rigotti 2007). It has

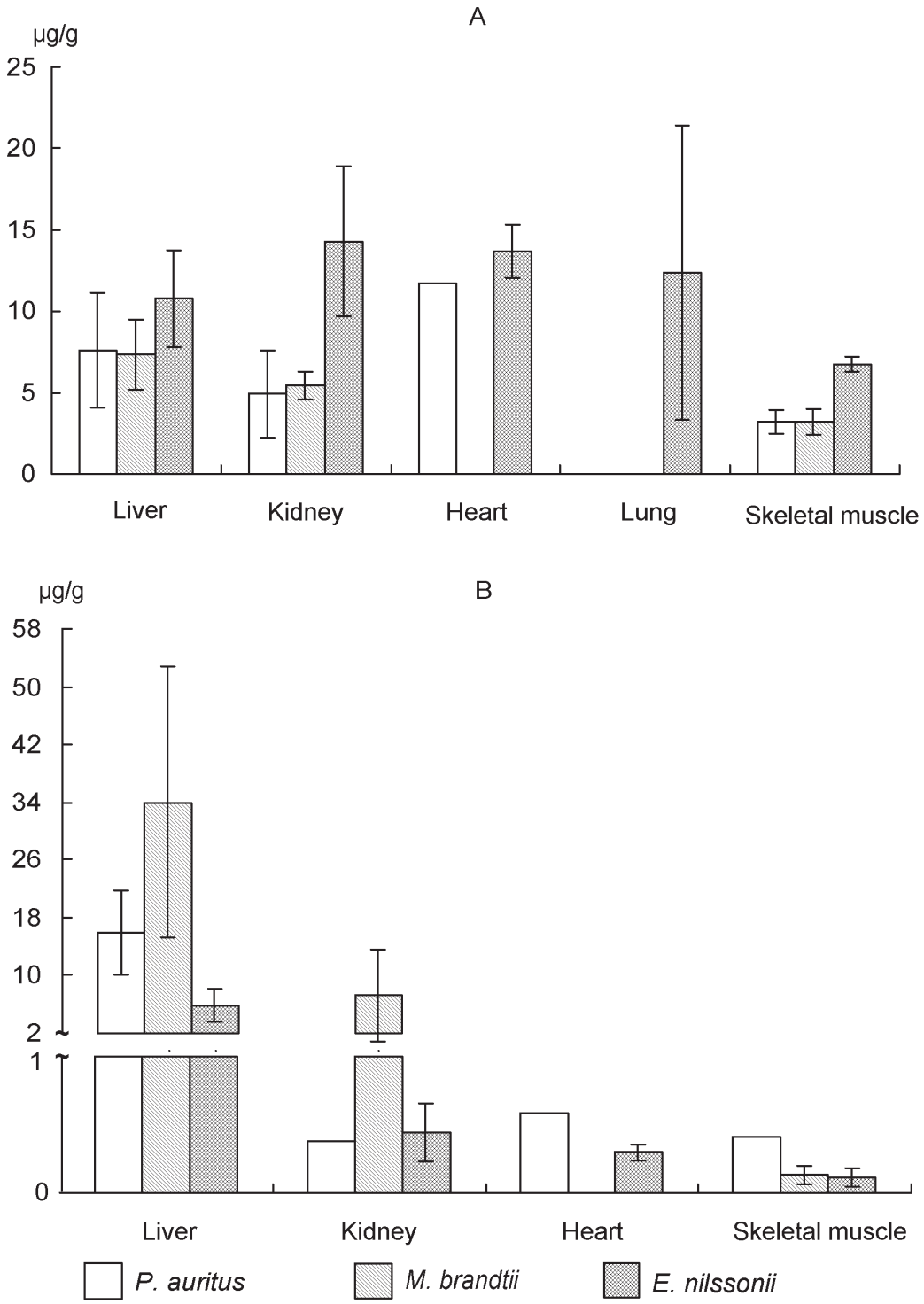

Fig. 4. α-Tocopherol (A) and retinol (B) content in bat tissues (means ± s.e.m.).

been reported that vitamin E accumulation in animal tissues in autumn may induce hibernation (Калабухов 1985). The northern bats that lived in colder areas with a shorter photoperiod and longer hibernation had in general higher values of low-molecular natural antioxidants, such as vitamin E, than bats from warmer areas with less shortening of the photoperiod. In addition, adequate levels of tocopherol during hibernation are essential for reproduction in spring.

The results of total WBC count and differential leukocyte count in hibernating bats are shown in Fig. 5 and 6. They indicate that during hibernation the species typically had a very low leukocyte level and, hence, relatively low absolute numbers of all types of cells in peripheral blood, making morphological and cytochemical studies rather complicated.

The morphology of formed elements in bat blood smears under light microscope is typical of mammals. Erythrocytes were represented by mature forms only, some individuals featured microcytes. Immature erythroid cells – nucleated normoblasts, as well as polychromatophils were absent. In some studies erythrocyte count did not change during hibernation unlike leukocyte count (Bouma et al. 2010, Reznik et al. 1975), whereas in other studies erythrocyte numbers gradually decreased further into hibernation (Нуритдинов 1990). Most species do not suffer

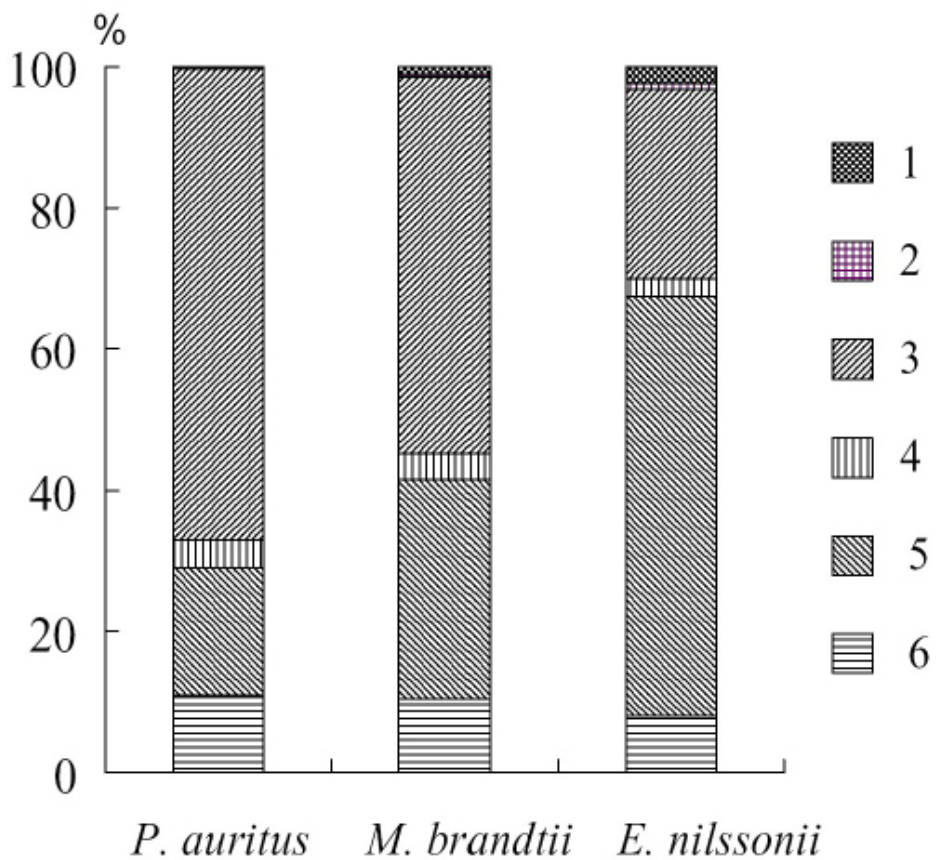

Fig. 5. Differential WBC count in different bat species.
1 - monocytes, 2 - lymphocytes, 3 - band neutrophils, 4 - segmented neutrophils, 5 - eosinophils, 6 - basophils.

hypoxia during hibernation in spite of a 10-fold and even greater decrease in the breathing rate. Meanwhile, the metabolic rate in the hypothermic state, for instance in the northern bat, dropped 210-270-fold compared to the active period (Ануфриев 2008). Apparently, the absence of morphological manifestations of bone marrow activation further proves that in the general context of considerable energy saving, reduced oxidative metabolism and some modified functions there is still a balance in the operation of physiological systems. Our results, indicating a low peripheral leukocyte count in hibernating bats, agree with data on other mammal species. In all small mammal species studied so far leukocyte numbers declined during hibernation (in the European ground squirrel e.g. by nearly 90%), and recovered rapidly after it (Bouma, Carey, Kroese 2010). A reduced amount of circulating leukocytes during hibernation as compared with active periods has been detected in the European hamster *Cricetus cricetus* (Linnaeus, 1758) (Reznik et al. 1975), the hedgehog *Erinaceus europaeus* (Linnaeus, 1758) (Suomalainen & Rosokivi 1973), as well as in ground squirrels – red-cheeked *Spermophilus erythrogenys* (Brandt, 1841) (Алексеева, Юнкер, Федорова 1974), European *Spermophilus citellus* (Linnaeus, 1758) (Bouma et al. 2010, Bouma, Carey, Kroese 2010), arctic *Spermophilus parryi* (Richardson, 1825) (Bouma et al. 2010, Bouma, Carey, Kroese 2010), long-tailed *Spermophilus undulates* (Pallas, 1779) (Ануфриев 2008) and 13-lined *Citellus tridecemlineatus* (Mitchill, 1821) (Spurrier, Dawe 1973). In the brown bear *Ursus arctos* (Linnaeus, 1758) leukocyte levels during hibernation are also lower compared to the active summer period, but unlike small mammals the bear maintains a relatively steady body temperature (Sahdo et al. 2013).

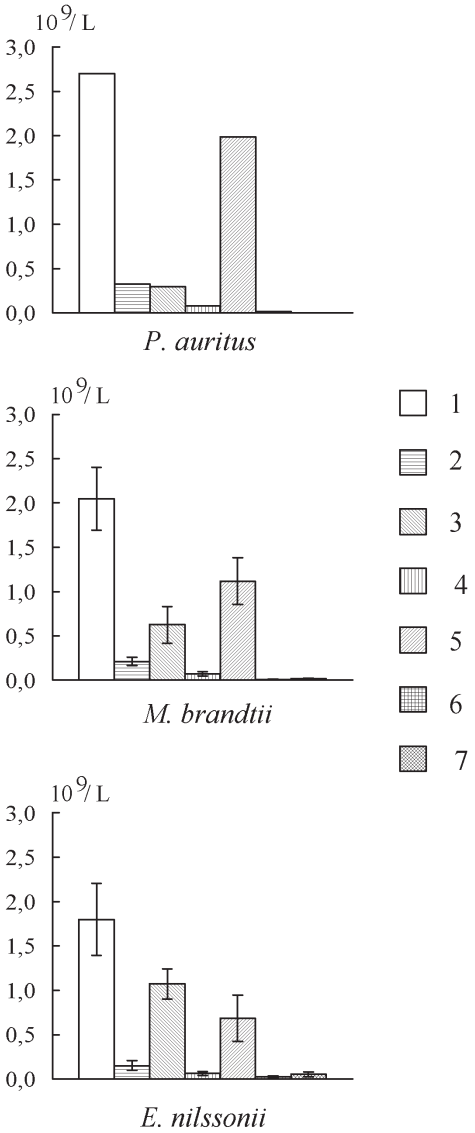

Fig. 6. Total WBC count and absolute numbers of each type of leukocytes in peripheral blood in bats.
1 - leukocytes 2 - monocytes, 3 - lymphocytes 4 - stab neutrophils, 5 - segmented neutrophils, 6 - eosinophils, 7 - basophils.

Regulation of peripheral blood composition during torpor has been insufficiently studied, and deserves to be scrutinized. Cold-induced thrombocyte/leukocyte platelet aggregation demonstrated in some *in vitro* experiments cannot explain the mechanism of reversible recovery to normal leukocyte numbers after arousal (Xavier et al. 2007). Some data suggest that the reduction in circulating blood volumes and leukocyte count during hypothermia is due to a redistribution of formed elements among organs and tissues, as well as to partial water loss (Ануфриев 2008). In bats, like in other

small mammalian hibernators, leucopenia affects all types of peripheral blood leukocytes – granulocytes, monocytes, and lymphocytes (Bouma, Carey, Kroese 2010). Band neutrophil numbers in the hedgehog declined during hibernation nearly 1.5-fold, and the decline in mature neutrophils was much greater – nearly 6-fold (Suomalainen & Rosokivi 1973). In the brown bear, no differences in lymphocyte numbers have been found between the active period and hibernation (Sahdo et al. 2013).

According to the literature, various hibernator species as a rule show a prevalence of neutrophils in peripheral blood (up to around 90%), whereas lymphocyte numbers are much lower – around 9% (Szilagyi & Senturia 1972). The species in our study differed in terms of leukocyte levels, as well as relative and absolute content of some leukocyte types in peripheral blood (Fig. 5 and 6). E.g., the brown long-eared bat and the Brandt's bat featured a relatively high level of neutrophilic leukocytes and, in contrast, a very low amount of lymphocytes. Lymphocyte counts in the northern bat in the study periods were higher than in the brown long-eared bat and the Brandt's bat.

Monocyte numbers in the bat species we studied were quite high. Other cell elements – basophils and eosinophils were either absent or scant, but the northern bat had more of them than the Brandt's bat and the brown long-eared bat (Fig. 5 and 6). In contrast to most other mammalian species, the northern bat had more basophils than eosinophils (Fig. 5). Basophils, which contain both clotting factors and hypocoagulation factors, apparently play an essential part in maintaining balance in the blood coagulation system during hibernation, alongside slower circulation and thrombocyte reduction.

CONCLUSIONS

It is in the eastern flanks of Fennoscandia, where the climatic conditions are the most severe (Republic of Karelia and Murmansk Region), and presumably in other areas north of 60° N that one can expect the lowest in Europe species richness and abundances of vesper bats. The dominant species among them, which stands out for a number of parameters (peripheral leukocyte and lymphocyte counts, LDH isoenzyme profile in some organs) is the northern bat. Compared to the Brandt's bat and the brown long-eared bat, it had a higher content of vitamin E in liver, apparently due to different availability of α-tocopherol in the end of hibernation, before the breeding season. Being resistant to low temperatures, the northern bat can remain active until late autumn, spend the winter in frost-prone caves unsuitable for other bat species, roost mainly on open ceiling and wall surfaces, and make little use of micro shelters (Ануфриев 2008, Рыков 2008, Белкин et al. 2013). The northern bat hibernates at lower temperatures (2.0±0.1ºC) and relative humidity (78.0±0.6%) than other species – Brandt's bat, whiskered bat, Daubenton's bat, as well as brown long-eared bat (Siivonen & Wermundsen 2008). On top of that, the northern bat features longer average torpor bouts compared to the brown long-eared bat and the Daubenton's bat (Ануфриев 2008). The relatively high peripheral lymphocyte count in hibernating northern bats must be a species-specific trait reflecting their physiological-biochemical characteristics.

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