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Some Differences in Thymus Morphology in Immature Insectivorous Mammals: *Sorex araneus*, *Sorex caecutiens*, *Neomys fodiens*, and *Erinaceus roumanicus*

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Abstract—The thymus structure of four species of immature insectivorous mammals belonging to the families Soricidae and Erinaceidae (Eulipotyphla) has been studied. Representatives of the studied families are characterized by contrary surviving strategies, significantly differing in the intensity of metabolism and the activity of the animal. As these differences are presumably reflected in the morphological parameters of the thymus, this work aimed at a comparative study of these parameters of the thymus in representatives of the above two families. A light microscopy technique has been applied. Sections of thymus lobes 5 μm thick have been stained with hematoxylin and eosin, as well as picrofuchsin, according to van Gieson's method, and with azure-eosin, according to Romanovskii–Giemsa. During the processing of the material, the mass index and the cortical-medullar index of the thymus have been determined. The area of the connective and lymphoid tissues on the thymus section has been identified. The number of thymocytes, as well as the number and area of the vessels of the microvasculature of both the thymus cortex and the medulla have been counted within the conventional unit area. The percentage of thymocytes in mitosis has been calculated. The results of the study show that the shrews have a higher thymus mass index than the northern white-breasted hedgehog. This leads to significant changes in the syntopy of the thymic lobules. In comparison to the northern white-breasted hedgehog, the thymus of shrews is characterized by an increased cortical-medullar index, a higher density of the arrangement of thymocytes per unit area, and a higher number and relative area of the vessels of the microvasculature. At the same time, all studied immature representatives of insectivorous mammalian species have equally high relative areas of lymphoid tissue. This indicates an active functional state of the thymus at this life stage in all Eulipotyphla representatives. The relative area of the thymus connective tissue is directly related to the absolute dimensions of the organ, which is necessary for the implementation of the frame function. The values of the mitotic index of the thymus medulla in Eulipotyphla representatives are higher than expected and may indicate the need for increasing the pool of thymocytes at a very late stage of differentiation. The patterns revealed indicate that the morphology of the thymus depends on the biological characteristics of representatives of different families of Eulipotyphla, has a certain adaptive value, and deserves further study.

Keywords: comparative morphology, thymus microvasculature, cortex and medulla, thymocyte density, thymocyte mitotic index

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INTRODUCTION

Representatives of the order Eulipotyphla are among the most numerous in vertebrate fauna and are characterized by a very high adaptive and competitive potential (Sundel et al., 2012; Eckrich et al., 2018). However, they vary significantly in the specific features of their adaptive strategies that ensure the maintenance of the internal environment of the organism (Kiselev, 2017; Rutovskaya et al., 2019; Haigh et al., 2012; Lazaro et al., 2019; Schaeffer et al., 2020). These differences cause inevitable restructuring of regulatory processes in the neuroimmunoendocrine system and contribute to the transformation of the morphological and functional state of the central organs of the lymphoid system ensuring the formation of an adequate and effective immune response (Kvetnoi et al., 2005; Prendergast et al., 2002; Gennen, 2012; Andersson and Tracey, 2012; Francini and Ottaviani, 2017; Whiting et al., 2018). A detailed comparative morphological study of the thymus for various representatives of the order Eulipotyphla provides better understanding the variability of the main characteristics of its standard morpho-functional organization. However, an analysis of the available scientific literature has shown that comparative morphological studies of the thymus of these mammals are extremely rare, and the available data do not allow us to form a holistic view on this issue. For this reason, the aim of this research is to study the structural features of the thymus in immature representatives of various species of the order Eulipotyphla, which differ in specific characteristics of their biology.

MATERIALS AND METHODS

Representatives of various species of the order Eulipotyphla (previously, Insectivora) that differ in key characteristics of their biology, i.e., body size, habitat, and metabolic rate (Kiselev, 2017; Rutovskaya et al., 2019; Haigh et al., 2012; Sundel et al., 2012; Eckrich et al., 2018; Schaeffer et al., 2020) have been studied. The indicators listed can affect the body in general and the lymphoid system in particular and, concordantly, can be reflected in their structure (Panov and Karpenko, 2004; Yurchinsky and Erofeeva, 2020; Käkelä and Hyvärinen, 1995). The morphology of the thymus was studied in immature representatives of four species of Eulipotyphla: the common shrew (*Sorex araneus* Linnaeus 1758, $n = 24$), Laxmann's shrew (*S. caecutiens* Laxmann 1788; $n = 16$), the Eurasian water shrew (*Neomys fodiens* Pennant 1771; $n = 12$), and the northern white-breasted hedgehog (*Erinaceus roumanicus* Barrett-Hamilton 1900; $n = 8$). The studied immature common shrews, Laxmann's shrews, and water shrews were of 2–3 months old and the underyearlings of the northern white-breasted hedgehog were of 1.5–2 months. The age of the animals was determined according to generally accepted methods (Klevezal, 2007). Animals were captured on the territory of Smolensk oblast, in natural biotopes, characterized by low anthropogenic pressure or its absence. In order to exclude the influence of unfavorable stress factors of the cold period of the year on the morphology of the thymus, the collection of material for all species studied was carried out from early June to mid-July.

Animal euthanasia was carried out by an overdose of ether anesthesia (ZAO Vekton, Russia) in accordance with the requirements approved by the order of the Presidium of the USSR Academy of Sciences of April 2, 1980, no. 12000-496 and the order of the Ministry of Higher Education of the USSR of September 13, 1984, no. 22, as well as the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The thymus was removed immediately after euthanasia, and then weighed and measured. Thymus segments were fixed in 10% neutral formalin, dehydrated, and embedded in paraffin according to the standard procedure. Thymus sections (5 μm) were obtained in the sagittal and horizontal planes on an HM 430 sledge microtome (Thermo Shandon Limited; serial no. 31144), stained with hematoxylin and eosin, picrofuchsin according to the van Gieson's method, and azure-eosin according to Romanovskii–Giemsa (Merkulov, 1969). The slides were photographed using a Sony ToupCam 5.1 imaging system (ToupTek, China) installed on a Micromed-3 Professional microscope (Micromed, China). Morphometric studies were performed on digital images of thymus preparations using the ToupView licensed image processing application (ToupTek, China). To assess the morphofunctional state of the thymus, the thymus mass index (TMI) was determined as the ratio of the mass of the organ to the body mass of the animal. The total area of the histological preparation of the thymus was measured at a magnification of the eyepiece ×5, and of the objective ×4. The areas of the thymus cortex and medulla were measured (in μm^2), and the cortical–medullar index was calculated as the ratio of the area of the cortex to that of the medulla. The total number of thymocytes in the cortex and medulla of the thymus was counted on a conventional unit area of 10 000 μm^2 (eyepiece ×10, objective ×90, under oil immersion) on digital photographs. For each preparation, ten visual fields were recorded. The number of mitoses in the subcapsular zone of the cortex and the medulla (mitotic index) was determined per 1000 registered cells (eyepiece ×15, objective ×90, under oil immersion). During the total study of the entire area of the preparation (with magnification of the eyepiece ×7, objective ×20), the total area occupied by the vascular bed, fibrous connective tissue, adipose tissue, and lymphoid tissue on the section was measured and determined (in % relative to the section area). In order to study the differences in the thymus medulla and cortex blood supply, the number and area of the microvasculature vessels (μm^2) were calculated on the conventional unit area (0.5 mm^2). Sections for vessel counting were randomly selected. On each preparation, vessels on ten such sections were counted. The identification of the links of the microvascular bed was based on generally accepted classifications (Kupriyanov, 1969). Microvessels include arterioles, precapillary arterioles, capillaries, and postcapillary venules (eyepiece × 15, objective × 20; × 40).

The statistical methods were implemented for the results obtained. The significance of differences between the compared groups was assessed by nonparametric (Mann–Whitney U-test and Kruskal–Wallis test) and parametric statistics (Student's *t*-test). The normality of the distribution of signs for was analyzed by the Lilliefors and Shapiro-Wilk tests, and the condition for the equality of the sample variances was checked using Levene's test.

RESULTS

According to the results obtained, members of different families of the order Eulipotyphla differ significantly in shape, size, and syntopy of the thymus lobes.

Thus, the representatives of the family Soricidae (Fischer von Waldheim, 1817), i.e., the common shrew, Laxmann's shrew, and the Eurasian water shrew, have thymus lobes increased in length and width. Furthermore, each lobe has a pronounced leaf-like shape (Fig. 1*C*). On the contrary, the thymus lobes of the northern white-breasted hedgehog (family Erinaceidae Fischer von Waldheim, 1817) were shortened, thickened, and widened in the region of the middle third, while narrowing cranially and caudally, which leads to the appearance of an ellipse (Fig. 1*D*). These differences cause discrepancies in thymic syntopy parameters. Thus, the thymus lobes of the white-breasted hedgehog are located exclusively in the region of the upper mediastinum. The cranial poles of the lobes reach the level of the lower cervical vertebra, while the lower poles reach the coronal sulcus of the heart and only slightly affect the upper part of its ventricles. In this case, the thymus lobes lie strictly in the plane of the sternal surface of the pericardium (Fig. 1*B*). On the contrary, in all representatives of the family Soricidae studied, the thymus lobes completely cover the heart, reaching its apex with their caudal poles, and make contact with the diaphragm. At the same time, due to a significant increase in width, the thymus lobes cover the pericardium in the form of a sheath, entering the slit-like spaces between the mediastinal surface of the heart and lungs (Fig. 1*A*). Thus, the thymus lobes in the white-breasted hedgehog are located only in the upper mediastinum, while in soricids they occupy a wider area, being in both the upper and lower mediastinum. In the region of the inferior mediastinum, the lateral edges of the strongly dilated thymus lobes of shrews fold sagittally and, as a result, go deep into the middle part of the inferior mediastinum, located between the mediastinal pleura and the pericardium. More than that, the thymus mass index in the white-breasted hedgehog is more than three times lower than that in shrews (Table 1). At the same time, the absolute mass of thymus lobes of the studied hedgehog reaches 0.45 ± 0.06 g, which is significantly higher than the corresponding indicators of the thymus of the water shrew (0.11 ± 0.09 g), as well as Laxmann's shrew (0.046 ± 0.06 g) and the common shrew (0.047 ± 0.05 g). The differences in the thymus weight of the water shrew and the members of the genus *Sorex* are statistically significant ($p < 0.05$, Mann–Whitney U-test and Kruskal–Wallis test). Being a member of the same order, the white-breasted hedgehog differs significantly from the studied shrews in some indicators characterizing the tissue structure of the thymus. For example, the cortical–medullar index of the hedgehog is less than one and a half times that of the shrew (Table 1; Figs. 2*C*, 2*D*). At the same time, on a conventional unit of area of the thymus cortex of 10 000 μm^2, the number of thymocytes for the hedgehog was 200 less than that of shrews (Table 1; Figs. 2*A*, 2*B*). However, the studied species differed significantly neither by the number of thymocytes in the thymus medulla, nor by the mitotic index of thymocytes in the cortex and medulla (Table 1). In all studied immature representatives of the order Eulipotyphla, the percentage of lymphoid tissue was equally high (Table 1).

The Eulipotyphla species studied differ in the content (%) of fibrous connective tissue in the thymus. Thus, in the thymus of the white-breasted hedgehog, the proportion of the area of this tissue on the cut was 3–4 times higher than that of the shrews. However, in the thymus of the water shrew, the content of fibrous connective tissue was statistically significantly higher only in comparison with the common shrew (Table 1). Comparison of the relative area of the blood vessels on the thymus sections showed that the thymus bloodstream is equally well developed in all the representatives of the family Soricidae studied. More than that, its relative area indicators are 3–3.5 times higher than the corresponding values determined for the white-breasted hedgehog (Table 1).

Of particular importance for the functional activity of the thymus is the development of the microcirculatory bed, the relative area of which is increased in all representatives of the family Soricidae and exceeds (by 1.3–1.8 times) the parameters of the thymus of the white-breasted hedgehog both in the cortex and in the medulla of the thymus (Table 1). Among the soricids studied, the Eurasian water shrew has the most developed microcirculatory bed of the thymus cortex and medulla, the parameters of which are statistically significantly higher than the corresponding values of the microcirculatory bed in the thymus of the common shrew and Laxmann's shrew (Table 1). In addition, the northern white-breasted hedgehog has fewer vessels in the microvasculature of both the cortex and the thymus medulla; this difference is statistically significant when comparing this species with the water shrew and the common shrew. In turn, the maximum values of these indicators were found in the water shrew. For example, the number of microcirculatory vessels in the thymus cortex and medulla of the Eurasian water shrew exceeds that of the common shrew and the white-breasted hedgehog by 1.5–2 times (Table 1).

DISCUSSION

According to the percentage ratio of various tissues in the thymus structure of the immature Eulipotyphla representatives studied, the lymphoid tissue dominates in the organ. This indicates the preservation of high thymus activity in all animals within a given age period, regardless of their taxonomy and biology (Sapin and Etingen, 1996; Pearse, 2006). The relative area of connective tissue in the thymus of the white-breasted hedgehog is significantly higher than that of the shrews. The thymus mass of the hedgehog is also several times greater than that of the shrews, which requires enhanced development of the connective tissue carcass. The correlation between the thymus mass and the amount of connective tissue in it was shown on

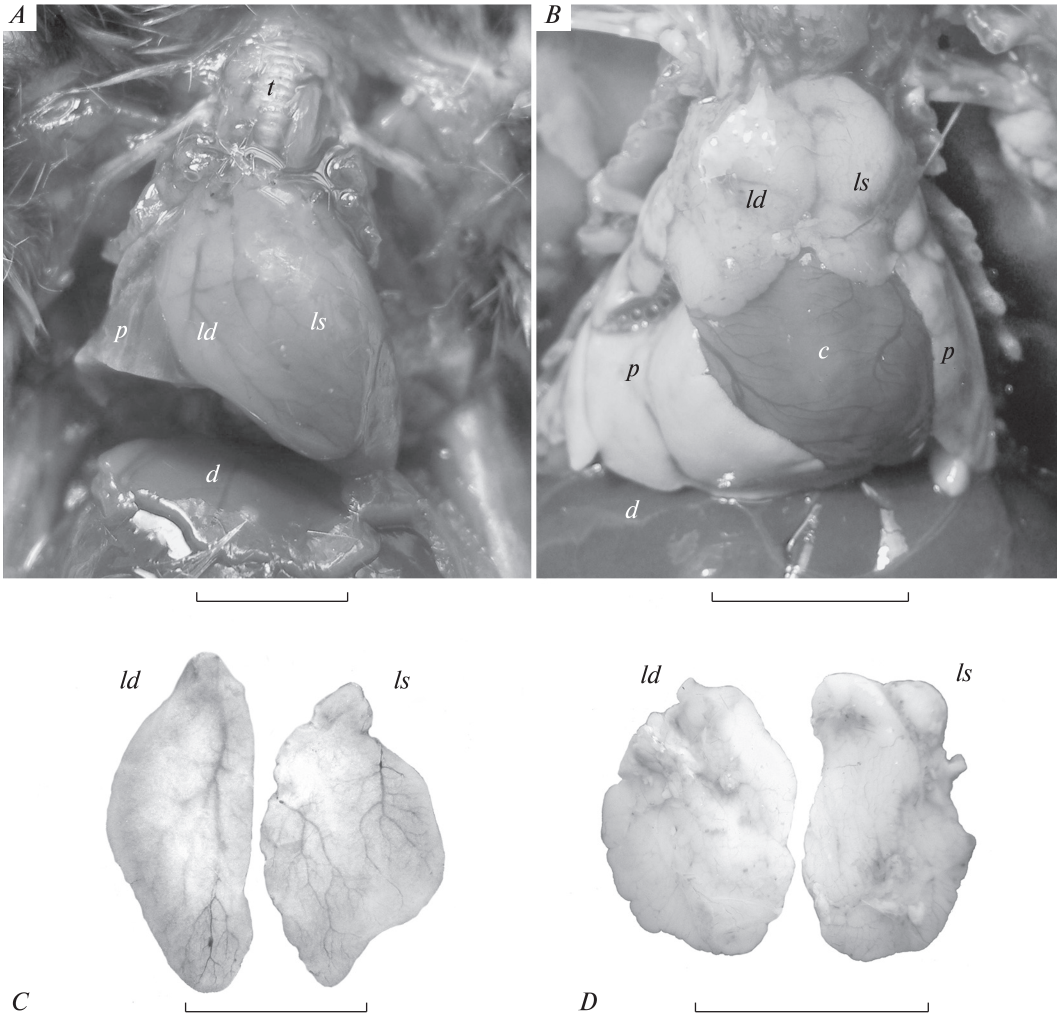

Fig. 1. Thymus macromorphology of the studied members of the order Eulipotyphla: *A*, *C*, thymus lobes of the common shrew; *B*, *D*, thymus lobes of the northern white-breasted hedgehog; *p*, lung; *t*, trachea; *c*, heart; *d*, diaphragm; *ld*, right thymus lobe; *ls,* left thymus lobe. Scale: *A*, *C*, 0.5 cm; *B*, *D,* 1 cm.

the example of various representatives of Chordata (Yurchinsky and Erofeeva, 2020). For the same reason, the amount of fibrous connective tissue in the thymus of the water shrew is higher than that of terrestrial shrew species.

A necessary condition for the survival of any organism is immune homeostasis, which is maintained in each group of animals taking into account different adaptive strategies (McDade, 2003; Long and Nathakumar, 2004; Andersson and Tracey, 2012). Eulipotyphla mammals are no exception, which is reflected in the morphology of the thymus, one of the central organs of the lymphoid system.

The most significant differences in the biology are between the studied representatives of different families of Eulipotyphla. In particular, the survival of representatives of the family Soricidae is based on the activation of motor activity and an increase in the metabolic rate (Kiselev, 2017; Rutovskaya et al., 2019; Haigh et al., 2012; Lazaro et al., 2019; Schaeffer et al., 2020). On the contrary, representatives of the family Erinaceidae chose to save energy, which is based on the formation of protective structures and a decrease in mobility (Chernova, 2005). These differences may affect the processes of lymphopoiesis in the red bone marrow and the rate of entry of bone marrow precursors into the thymus cortex, which are highly sensitive to various external and internal environmental factors (Bhandoola et al., 2007; Mori et al., 2007; Dudakov et al., 2010). These facts are confirmed by the experimental data of other authors, proving that the formation of immunocompetence is an energetically expensive process, and the energy resources necessary for its provision appear as a result of their adaptive redistribution according to the tradeoff principle to the detriment of other vital functions (Long and Nanthakumar, 2004; Abrams and Miller, 2011; Wang et al., 2019). For these reasons, the energetically more eco-

Table 1. Main characteristics of thymus morphology in the members of the order Eulipotyphla studied ($X \pm sx$)

Characteristics	Common shrew	Laxmann's shrew	Eurasian water shrew	Northern white-breasted hedgehog
Thymus mass index (TMI) Mann–Whitney U-test, $p < 0.05$ Kruskal–Wallis test, $p < 0.05$	0.65 ± 0.07^{d}	0.63 ± 0.07^{d}	0.61 ± 0.05^{d}	$0.18 \pm 0.01^{a, b, c}$
Cortical–medullar index (CMI) Mann–Whitney U-test, $p < 0.05$ Kruskal–Wallis test, $p < 0.05$	7.13 ± 0.83^{d}	7.03 ± 0.75^{d}	7.32 ± 0.80^{d}	$4.36 \pm 0.60^{a, b, c}$
Number of thymocytes ($S = 10000\ \mu m^2$) Student's t-test, $p < 0.05$				
cortex	776.32 ± 63.26^{d}	742.11 ± 58.72^{d}	759.43 ± 55.47^{d}	$544.39 \pm 46.38^{a, b, c}$
medulla	366.03 ± 31.64	343.92 ± 29.78	358.94 ± 27.49	334.68 ± 29.47
Mitotic index (% per 1000 thymocytes) Student's t-test, $p \geq 0.05$				
cortex	3.62 ± 0.35	3.93 ± 0.33	4.15 ± 0.36	3.46 ± 0.30
thymus medulla	3.02 ± 0.40	3.21 ± 0.35	3.11 ± 0.30	3.05 ± 0.32
Area (%) of tissue Student's t-test, $p \geq 0.05$				
lymphoid	97.66 ± 1.09	97.68 ± 0.43	97.32 ± 0.55	98.19 ± 0.69
fibrous connective	0.38 ± 0.05^{d}	0.33 ± 0.04^{d}	$0.47 \pm 0.04^{b, d}$	$1.26 \pm 0.09^{a, b, c}$
Vascular bed area (%) Student's t-test, $p \leq 0.05$	2.08 ± 0.14^{d}	1.99 ± 0.10^{d}	2.19 ± 0.12^{d}	$0.61 \pm 0.04^{a, b, c}$
Vessel area (%) of the microvasculature Student's t-test, $p \geq 0.05$				
cortex	0.0091 ± 0.0007^{d}	$0.0077 \pm 0.0006^{c, d}$	$0.0108 \pm 0.0008^{b, d}$	$0.0061 \pm 0.0004^{a, b, c}$
medulla	$0.0147 \pm 0.0011^{b, d}$	0.0122 ± 0.0009^{c}	$0.0170 \pm 0.0012^{b, d}$	$0.0108 \pm 0.0009^{a, c}$
Number of microvasculature vessels ($S = 0.5\ mm^2$) Student's t-test, $p \leq 0.05$				
cortex	$32.71 \pm 2.68^{c, d}$	$26.92 \pm 2.19^{c, d}$	$40.73 \pm 4.11^{a, b, d}$	$22.44 \pm 3.11^{a, c}$
medulla	$39.36 \pm 3.01^{c, d}$	33.57 ± 3.73^{c}	$47.23 \pm 3.42^{b, d}$	$30.92 \pm 2.18^{a, c}$

* Significance of age differences ($p \leq 0.05$), significance of differences ($p \leq 0.05$) in comparison with: [a], the common shrew; [b], Laxmann's shrew; [c], the Eurasian water shrew; [d], the northern white-breasted hedgehog.

nomical (compared to shrews) adaptive strategy of the northern white-breasted hedgehog allows us to maintain immune barriers in an optimal state under conditions of a significant decrease in the influx of T-lymphocyte precursors into the thymus cortex, which results from a slowdown in the rate of formation of lymphoid cells in the red bone marrow. Later, this prompts the appearance of a cascade of morphological differences in the hedgehog thymus in comparison with shrews. In the hedgehog thymus, the organ mass index, the density of the arrangement of lymphocytes in the cortex, and the index of its relative area are

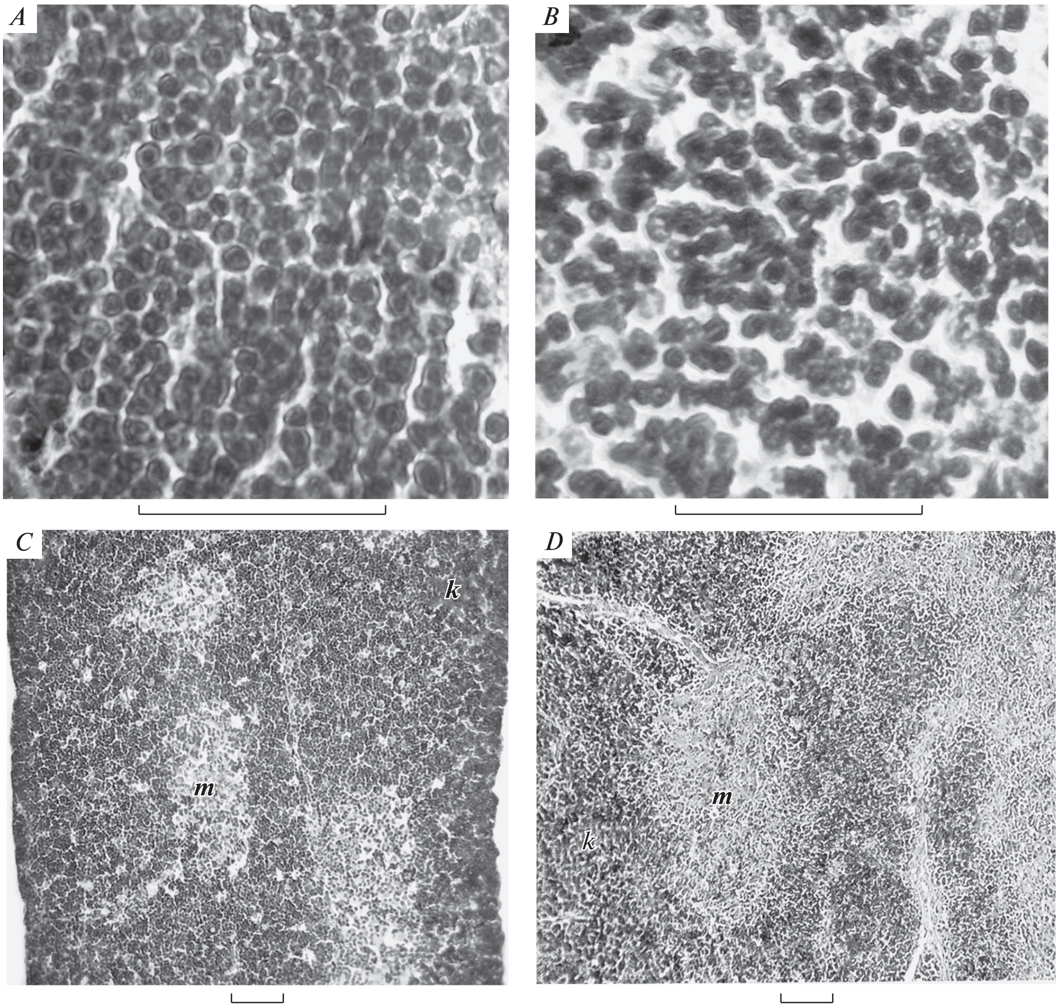

Fig. 2. Thymus micromorphology of the studied members of the order Eulipotyphla. Density of thymocytes in the thymus cortex of the common shrew (*A*) and the northern white-breasted hedgehog (*B*). Cortex and medulla on a section of the thymus of the common shrew (*C*) and the northern white-breasted hedgehog (*D*): *m*, medulla; *c*, cortex. Scale 50 μm.

reduced, as evidenced by the decrease in the value of the cortical–medullar index. All this highlights the ability of the hedgehog body to maintain its immune status despite a reduced number of lymphoid cells entering the thymus and concentrated in it.

There is no doubt that there is a close dependence between the morphological parameters of the circulatory system and the characteristics of biological processes in tissues and organs (Galagudza et al., 2016; Meyer, 2018). In particular, a direct relationship has been found between the development of the microvasculature and the intensity of cell proliferation in the tissues of lymphoid organs (Balu, 1977). The determined increase in the indicators of various parameters of the bloodstream in the thymus of shrews (compared with the northern white-breasted hedgehog) indicates a higher functional activity of this organ. It can be assumed that the increased motor activity of representatives of the family Soricidae causes an increase in the probability of encountering pathogens, which requires an adaptive strengthening of immune barriers. At the same time, a higher level of metabolism of these animals predetermines the ability to make more significant investments in the immune system compared to the similar abilities of the organism of the white-breasted hedgehog, which results in the functional activation of the lymphoid system in general and the thymus in particular.

A number of studies indicate the existence of biological differences between representatives of the genus *Sorex* and the genus *Neomys* associated with adaptation to the conditions of either a terrestrial or near-aquatic lifestyle. In particular, it was found that, in comparison with terrestrial shrews, the water shrew, inhabiting the zone of the edge effect, has contact with a wider range of parasite fauna species, which inevitably puts additional pressure on its immune system (Panov and Karpenko, 2004). According to experimental data, differences in the food objects cause differences in the metabolism of terrestrial and semiaquatic shrews (Käkelä and Hyvärinen, 1995). Compared to terrestrial shrews, water shrews have developed physiological mechanisms that ensure the appearance of neurotoxins in saliva (Kowalski et al., 2017). As our results show, significant differences in biology associated with the near-aquatic environment are also reflected in the morphology of the thymus. The near-aquatic lifestyle, first of all, affects the parameters of the vessels of the thymus microvasculature of the water shrew, increasing their number and relative area, both in the cortex and in the medulla of the thymus.

The thymus cortex is characterized by very high rates of maturation and proliferation of thymocyte precursors (Le Campionet al., 2000). At very late stages of differentiation, single positive thymocytes that have undergone negative selection processes are concentrated in the thymus medulla (Chen, 2004; Luc et al., 2007). Increased indices of the mitotic index of thymocytes in the thymus medulla of the studied Eulipotyphla indicate active processes of negative selection in the thymic medulla and an increase in the pool of tolerant naive thymocytes through their mitotic division (Le Campion et al., 2000; Chen, 2004; Klein et al., 2009).

Thus, the influence of the biological characteristics of species on some processes in the lymphoid system has been shown on the example of members of Eulipotyphla. This is reflected in the morphology of their thymus. In particular, it has been found that the activation of metabolism, which results from the activation of vital activity in shrews, is associated with an increase in the processes of formation, transport, and proliferation of thymocyte precursors. Therefore, indicators of a number of key morphological characteristics of the thymus (such as the mass index, cortical–medullar index, and mitotic index) increase. The density of thymocytes in the cortical substance of the organ increases, and the indices of the microcirculatory bed also become higher. A different strategy, typical for the white-breasted hedgehog, on the contrary, is associated with a more economical use of energy and is accompanied by diametrically opposite changes in the above morphological characteristics of the thymus. In other words, the variability of the morphological characteristics of the thymus indicates that this organ is actively involved in the processes of maintaining immune homeostasis through an adaptive change in the number of immunocompetent T-lymphocytes entering the bloodstream (Kondo et al., 2019).

The studied species of the order Eulipotyphla did not differ in the relative amount of lymphoid tissue in the thymus; however, all these species were characterized by the dependence of the quantitative characteristics of the fibrous connective tissue on the size of the thymus.

The revealed patterns have a certain adaptive value and merit further study.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The author declares that he has no conflicts of interest.

Statement on the Welfare of Animals

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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