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PECULAR FEATURES OF HEMATOPOIESIS IN THE LIVER OF MATURE AND IMMATURE GREEN FROGS (*PELOPHYLAX ESCULENTUS* COMPLEX)

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Peculiar Features of Hematopoiesis in the Liver of Mature and Immature Green Frogs (*Pelophylax esculentus* complex). Akulenko, N. M. — The article describes characteristic features of the hematopoiesis in mature and immature green frogs (*Pelophylax esculentus* complex). Quantitative differences in liver myelograms were insignificant. However, in a sample of mature animals numerous significant correlations between the number of pigment inclusions in the liver and indicators of erythropoiesis and myelopoiesis were observed. Those correlations were absent in the immature frogs. We concluded that after the frogs' breeding a lack of plastic resources, in particular, hemosiderin remains up to the hibernation.
K e y w o r d s: frog, pigment cells, hematopoiesis, reproduction.

Introduction

Hematopoietic processes of the frogs during embryonic and larval development and immediately after metamorphosis were the subject of rather detailed studies (Maniatis, Ingram, 1971; Smyslov, 1980; Meseguer et al., 1985; Rollins-Smith et al., 2004, etc.). The hematopoiesis of mature animals has been also the subject of many studies discussed in detail earlier (Akulenko, 2011, 2012). However, the breeding season is a severe stress for frogs;it causes the death of a large part of the animals in populations. The main cause of the stress is the exhaustion (Kuzmin, 1999). After the breeding and up to the hibernation, the body of mature animals restores, but is the restoration complete? How the consequences of the depletion may affect the blood formation processes? These questions were not the subjects of any special studies. If we consider the metabolic support of the hematopoiesis in amphibians, special attention should be paid to the functional characteristics of the pigment cells of the liver. Pigment cells of the liver and spleen in anuran amphibians are a variety of storage cells accumulating heme (in the form of hemosiderin) and melanin. There is an evidence that the pigment cells of amphibian liver are involved in the processes of non-specific defense of the body (Akulenko, 1998; Agius, Roberts, 2003; Fenoglio et al., 2005), in the processes of liver regeneration in response to toxic damage (Akulenko, 2009), in erythropoietic processes including the compensatory one (Akulenko, 2010). Data on the metabolism of the liver pigments allow to judge about the general metabolic processes of hematopoiesis, at least about its erythroid and granulocytic germ. Thus, our aim was to examine the processes of hematopoiesis in immature representatives of anurans and compare them with similar processes in mature individuals from the same population. We also compared the functional activity characteristics of liver pigment cells which may indicate the metabolism of the hemoglobin and many oxidative metabolic enzymes.

Material and methods

Studies were carried out on the individuals of green frogs (*Pelophylax esculentus* complex) from the agrocenosis near the village Erchiki on the bank of the Unava River (Zhytomyr Region, Ukraine). As an experimental group we selected the individuals that had passed through the metamorphosis a year ago and possessed the hematopoiesis rhythm characteristic for adult individuals; they did not undergo, however, a period of exhaustion during the development of sexual products and mating. Thus, the state of the hematopoietic system in these individuals may be conventionally taken as the standard of the normal process of hematopoiesis in the definitive state. We compared a sample of 8 immature animals (the body length 2.4–3.6 cm, weight 2.5–4.6 g). and a sample of adult animals (27 males and females, weight 12–50 g, body length 6–12 cm) from the same biotope. Nine males of *Pelophylax ridibundus* (weight 40–50 g, body length 8–10 cm) collected from a natural habitat were the control group. In addition, we identified a sample of mature individuals with strong liver hyperpigmentation and calculated all the studied parameters for them separately.

The amount of pigment inclusions and indicators of the hematopoiesis depends on the season (Akulenko, 1998; Barni, 2002), therefore, in all cases we studied the animals caught in August and September, when sexually mature animals recover after reproduction, and hematopoiesis is maximal (Akulenko, 2011). Count of cells involved in the hematopoiesis was made on liver smears. For each animal, we counted the myelogram and made calculations and descriptions of the macrophage lineage cells: macrophages, monocytes, and pigment cells by previously developed methodology (Akulenko, 1998). For each animal, we calculated indicators describing the functional activity of macrophages and the regeneration rate of pigment cells (table 3). For analysis of the results for each sample we calculated average values, errors, and significance of differences between the control and the experimental samples. Also, the correlation coefficients were calculated for each indicator with the total amount of pigment in the liver (tables 1–3). Calculations were performed using Microsoft Excel following the previously developed technique (Akulenko, 2011). Number of pigment inclusions in the liver was determined on sections where the percentage of the area occupied by pigment cells was calculated using the eyepiece grid.

Results and discussion

Previous studies on the green frog hematopoietic system showed that it comprises two units: the central or the basic one — the bone marrow, and the peripheral one consisting of the liver, the spleen and the blood stream. Hematopoiesis in the bone marrow is a fairly constant process. It is only the content of myeloid tissue in the medullary cavity that changes, and the changes depend on the season. In the liver and in the blood stream, in contrast, differentiation of blood cells related to particular line ages is activated depending on the season (Akulenko, 2012). Thus the hematopoiesis in the bloodstream is more responsive to the individual needs of the animal, than blood formation in the liver. Considering all the above, we have decided that the formation of blood in the liver will be the most suitable model for the study of differences between sexually mature and immature individuals.

Signs of intense erythropoiesis and myelopoiesis are present in the liver of immature frog (fig. 1). While comparing the myelogram indicators of immature and mature individuals from agrocenosis, we found most of the values to be practically the same (tables 1, 2).

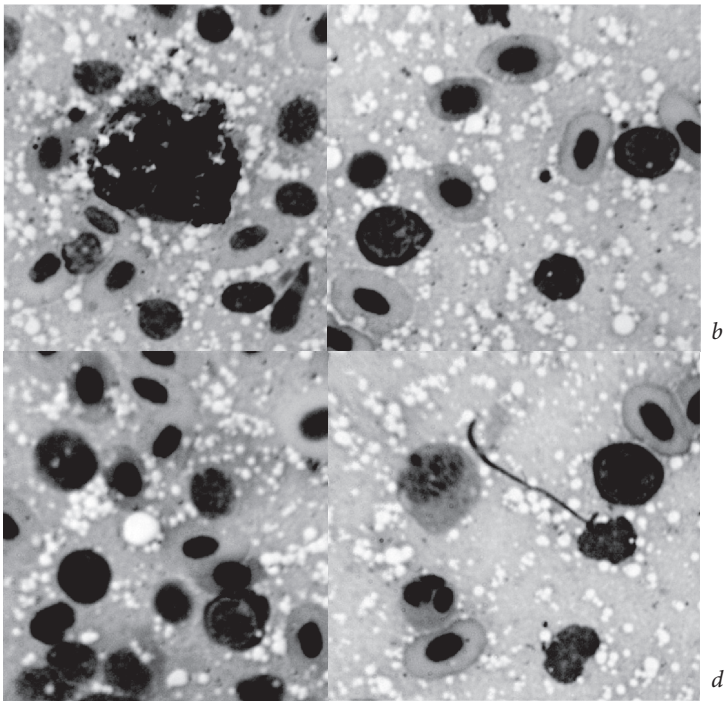

Fig. 1. Smear-imprint of the liver of immature green frog: *a* — pigment cells; *b* — erythroblasts; *c* — undifferentiated blast, erythroblast and eosinophilicmyelocyte; *d* — erythroblast and medullocell neutrophil. Pappenheim staining, ×200.

Table 1. Indicators of erythropoiesis and overall proliferative activity of cells in the liver of green frogs caught in agrocenosis (all in %, except for "ratio" indicator)

Indicators	Control		Immature		Mature		With a maximum content of pigment		The correlation coefficientof the index with an area of pigmented inclusions			Differences between immature individuals and mature ones with maximum content of pigment
	M	m	M	m	M	m	M	m	immature	mature	with a maximum content of pigment	
Immature erythroid cells (amount)	29	9.8	28	4.6	25	3.2	10.1	4.9	–0.5	-0.6 $p < 0.01$	–0.22	2.7 $p < 0.05$
Erythroblasts	2.9	0.6	3.5	0.5	3.4	0.6	1.4	0.7	–0.5	-0.6$p < 0.01$	–0.4	2.5 $p < 0.05$
Normoblasts basophilc	8.8	2.7	5.2	1	6.8	1.1	2.6	1.1	–0.3	-0.6 $p < 0.01$	–0.34	
Normoblasts polychrome	20	6.9	20	3.5	14	1.8	8.2	3.3	–0.4	-0.5 $p < 0.01$	–0.1	2.4 $p < 0.05$
Erythroblasts / normoblasts polychrome (ratio)	0.5	0.3	0.5	0.4	0.3	0.1	0.2	0.1	–	–	–	
Undifferentiated blasts	1.6	0.3	3.1	0.5	2.3	0.2	2.2	0.6				
Mitosis	0.2	0.1	0.9	0.2	0.7	0.2	0	0				3.7 $p < 0.01$

At the same time the both groups revealed a significant difference from the control sample collected in clean natural habitats. It can be concluded that the processes of hematopoiesis in the peripheral part of the hematopoietic system are equally intense in mature and immature individuals, and the impact of pollution in agrotcenos is on these processes does not differ significantly. However, if we consider the process of erythropoiesis in detail, we can see that in the immature frogs all indicators relating to it are higher and closer to the control, although the difference is insignificant. Those indicators were less dependent on the amount of pigment reserves in the liver than in the mature frogs (table 1). Noteworthy is also the ratio of "erythroblasts: polychrome normoblasts" in the studied groups of animals. In immature frogs and in the control group this ratio is around 0.5, indicating that a significant proportion of erythroblasts leave the liver for further differentiation in the bloodstream (Akulenko, 2011, 2012). In adults the ratio is 0.3, which means that less erythroblasts continue differentiation in the blood stream. Indicator "immature myeloid cells: mature (ratio)" demonstrated significant differences. In mature frogs it was significantly higher, indicating a lower proportion of mature granulocytes in the liver. The latter is most likely due to the depletion of the pool of mature granulocytes in the bloodstream. These discrepancies can be explained by assuming that in mature individuals seasonal differentiation of erythrocytes and granulocytes occurs under conditions of iron deficiency for synthesis of hemoglobin and hemosiderin. In individuals who do not participate in reproduction, the body is sufficiently saturated with heme compounds, and the lack of it is not a limiting factor for hematopoiesis. In immature animals with normal erythropoiesis surplus of heme is deposited in the liver in the form of hemosiderin. These reserves suffice for a complete synthesis of enzymes in the differentiation of granulocytes and macrophages. This statement is confirmed by the fact that in the conditions when there is excess of hemosiderin in the liver, "not working" are most of the correlations between the number of granulocytic cells and the content of pigments in the liver. Data in table 2 demonstrate many significant correlations between the amount of pigment deposits in the liver and the number of mature granulocytes in mature frogs. These correlations are absent in immature

Table 2. Indicators of myelopoiesis the green frogs caught in agrocenosis (all in %, except for "ratio" indicator)

Indicators	Control		Immature		Mature		With a maximum content of pigment		The correlation coefficientof the index with an area of pigmented inclusions		Reliability of differences between Immature and		
	M	m	M	m	M	m	M	m	immature	mature	mature	control	frogs with maximum content of pigment
Myeloidcells (amount)	35	4.3	18	2.4	16	1.1	14	2.7				3.4 p < 0.01	
Myeloblasts	0.8	0.2	0.9	0.4	1.3	0.6	1.2	0.4					
Neutrophilic myelocytes	1.6	0.4	0.5	0.2	1.1	1.1	1.4	0.6	0.8 p < 0.01	0.5 p < 0.01		2.4 p < 0.05	
Eosinophilic myelocytes	1.5	0.5	0.6	0.1	0.8	1.8	0.5	0.2					
Metamyelocytes neutrophilic	2.1	0.6	0.7	0.3	0.7	0.2	0.4	0.3				2 p < 0.1	
Metamyelocytes eosinophilic	1.7	0.4	0.7	0.2	1.5	0.3	2.5	0.6		0.7 p < 0.01	2.3 p < 0.05	2.3 p < 0.05	2.9 p < 0.05
Stab neutrophils	10	2.3	7.1	1.1	4.7	0.1	4.1	1.1			2.2 p < 0.05		1.9 p < 0.1
Stab eosinophils	5	1.5	1.4	0.5	1.8	0.2	1.3	0.4				2.3 p < 0.05	
Neutrophils-segmented	7.4	1.6	4.7	1.4	3.1	0.2	3.6	1.5					
Eosinophils segmented	7.7	1.2	1.7	0.2	1.6	0.6	2.3	1.1		0.5 p < 0.01		4.9 p < 0.01	
Basophils	9.3	1.4	3.4	0.7	3.1	0.6	4.1	1.7		0.5 p < 0.01		3.7 p < 0.01	
Immature myeloid cells	7.1	1.5	3.5	0.9	5.3	0.6	5.1	0.4		0.53 p < 0.01		2.1 p < 0.05	
Immature myeloid cells: mature (ratio)	0.3	0.1	0.2	0.1	0.7	0.1	0.7	0.3		0.34 p < 0.1	4.1 p < 0.01		
Immature neutrophils	3.8	1	1.8	0.6	2.4	0.5	1.5	0.7				1.8 p < 0.1	
Neutrophils mature	19	3.7	16	2.5	10	1.2	6.7	2.3			2.3 p < 0.05		2.9 p < 0.05
Eosinophilsim mature	3.4	0.6	1.9	0.4	2.9	0.4	2.5	0.4				2 p < 0.1	
Eosinophils mature	14	2.4	4.6	1.2	4.6	0.7	3.2	1.3				3.5 p < 0.01	
All stab	14	2.8	8.5	1.3	6.5	0.7	4.5	1.3				1.8 p < 0.1	2.2 p < 0.05
All segmented	14	2.6	6.4	1.3	4.7	0.7	4.9	1.8				2.6 p < 0.05	

specimens. The same applies to the functional activity of macrophages. Macrophage phagocytic activity in mature frogs depends on the presence of hemosiderin in the liver (table 3). In immature frogs the resource is there in abundance and these correlations do not work. Also immature specimens are missing numerous negative correlations between indicators of granulocyte differentiation and functional activity of macrophages (table 4). This can again be explained by sufficient amount of heme in the liver. Under this condition there is no competition for the resource between two cell lines. Indicators "pigment cells young" and "pigment cell with signs of the degranulation" do not differ in mature and immature

Table 3. Some indicators of the functional activity of pigment cells and macrophages in the liver of green frogs fromagrocenosis (all in %, except for "ratio" indicator)

Indicators	Control		Immature		Mature		Frogs with maximum content of pigment		The correlation coefficient of the index with an area of pigmented inclusions		Differences between Immature and		
	M	m	M	m	M	m	M	m	immature	mature	mature	Control	With a maximum content of pigment
Pigmentcells / macrophages ratio	1.20	0.35	0.8	0.2	1.4	0.4	3.6	1.4		0.7 $p < 0.01$			2 $p < 0.1$
Macrophages with inclusions (% of total)	56.81	7.03	66	4.7	67	3.3	83	5.3		0.5 $p < 0.01$			2.3 $p < 0.05$
Macrophages with phagocytosed pigment (% of total)	29.50	5.58	50	4.5	45	3.5	69	6.3	0.6 $p < 0.1$	0.6 $p < 0.01$		2.8 $p < 0.05$	2.5 $p < 0.05$
Macrophages with basophilic inclusions (% of total)	10.56	3.14	14	1.3	20	2.9	10	5			1.8 $p < 0.1$		
Young pigment cells (% of total)	4.48	1.74	14	3.9	17	2.7	17	4.9				2.2 $p < 0.05$	
Pigment cells with signs of the deganulation (% of total)	25.12	4.51	9.5	4.6	6.6	2.4	4.9	2				2.4 $p < 0.05$	
Area pigment inclusions in sections (% of the slice area)	1.58	0.5	3.35	0.45	2.01	0.48	7.1	0.9					

Table 4. The values of correlation coefficients of some indicators of myelopoiesis and phagocytic activity of macrophages in the liver of green frogs from agrocenosis

Indicators	The correlation coefficient	
	immature	mature
Myelocytes /macrophageswithinclusions	–0.2	–0.47 $p < 0.05$
Eosinophilicmyelocytes / macrophages with inclusions	–0.2	–0.38 $p < 0.05$
Neutrophilicmetamyelocytes/ macrophages with inclusions	–0.2	–0.49 $p < 0.01$
Eosinophilicmetamyelocytes / macrophages with basophilic inclusions	–0.2	–0.32 $p < 0.1$
Immature myeloid cells (amount)/ macrophages with inclusions	0	–0.47 $p < 0.05$
Immature neutrophils / macrophages with inclusions	0	–0.58 $p < 0.01$

animals from agrocenosis. The first indicator shows the differentiation rate in the population of pigmented cells, the second one increases when the rate of degradation slows down. However, all samples from agrocenosis significantly differed from the control.

Thus, we conclude that immature frogs show the optimal mode of a blood production, at which the differentiating cells are sufficiently provided by resources. On the contrary, even the survivors which participated in breeding have signs of anemia. Iron deficiency affects erythropoiesis and myelopoiesis, and during reactions of defense and recovery.

In order to check the degree of the differences between immature and mature individuals we also compared immature individuals and the sample of adult frogs with maximum content of pigments in the liver. As a working hypothesis it was assumed that the sample included individuals who were able to adequately recover after the breeding season, and their indicators might be close to optimal hematopoiesis. However, the assumption was not confirmed. In the individuals with hyperpigmentation the differentiation of erythroid cells was sharply reduced, the total number of neutrophils was significantly reduced, the number of macrophages in the liver and their functional activity was increased (tables 1–3). All these parameters reliably distinguish individuals with hyperpigmentation from immature frogs and from the control sample collected in clean habitats. Based on these data, we infer that an additional amount of pigment is not formed in the liver due to excessive accumulation of iron in the body, but due to inhibition of erythropoiesis. Thus, we conclude that the hyperpigmentation in adult frogs is not the norm, but rather a compensatory response to some yet unknown factors.

Conclusion

Significant quantitative differences in the processes of erythropoiesis and myelopoiesis in mature and immature specimens of green frogs are absent; however, in the immature specimens the hematopoiesis is not limited by the lack of plastic resources such as hemosiderin. It can be concluded that the majority of mature individuals leave for hibernation not fully recovered after taking part in reproduction.

References

Akulenko, N. M. 1998. Seasonal dynamics of amount and functional activity of macrophages and the pigment cells in the liver of the lake frog. *Vestnik Zoologii*, **32** (4), 86–93 [In Russian].

Akulenko, N. M. 2009. Amphibians as indicators of human toxicity: opportunities and challenges of the method. *Ekologicheskiy monitoring i bioraznoobraziye*, **4** (1–2), 140–147 [In Russian].

Akulenko, N. M. 2010. Modification of erythropoiesis in amphibians in anthropogenic landscapes. *Proceedings of the symposium "The morphogenesis of organs and tissues beneath.the influence of exogenous factors" 7–9 October 2010, the Simferopol–Alushta. Simferopopol, 2010*, 24–28 [In Russian].

Akulenko, N. M. 2011. Vascular bed as part of the hematopoietic system of anurans.*Vestnik Zoologii*, **45** (4), 359–366 [In Russian].

Akulenko, N. M. 2012. Haemopoietic system of the anurans: the role of bone marrow and liver. *Vestnik Zoologii*, **46** (4), 347–354.

Kuzmin, S. L. 1999. *Amphibians of the former USSR*. Association of scientific editions. KMK, Moscow, 1–298 [In Russian].

Smyslov, N. Y. 1980. *Experimental study of the development of hematopoietic tissue in embryogenesis of grass frog Rana temporaria.* Ph.D thesis. Moscow State University, 1–22 [In Russian].

Agius, C., Roberts, R. J. 2003. Melao-macrophage centres and their role in fish pathology. *J Fish Diseases*, **26** (9), 499–509.

Barni, S., Vaccarone, R., Bertone, V., Fraschini, A., Bernini, F., Fenoglio, C. 2002. Mechanisms of changes to the liver pigmentary component during the annual cycle (activity and hibernation) of *Rana esculenta* L. *J Anat*, **200** (2), 185–194.

Fenoglio, C., Boncompagni, E., Fasola, M., Gandini, C., Comizzoli, S., Milanesi, G., Barni, S. 2005. Effects of environmental pollution on the liver parenchymal cells and Kupffer-melanomacrophagic cells of the frog *Rana esculenta*. *Ecotoxicol Environ Safety*, **60** (3), 259–268.

Maniatis, G. M., Ingram, U. M. 1971. Erythropoiesis during amphibian metemorphosis. 1. Site of maturation of erythrocytes in *Ranacatesbeiana*. *J Cell Biol*, **49** (2), 372–379.

Meseguer, J., Lozano, M., Agulleiro, B. 1985. Ultrastructure of the renal granulopoietic tissue of the *Rana ridibunda* tadpole. *J Submicrosc Cytol*, **17** (3), 391–401.

Rollins-Smith, L. A., Hopkins, B. D., Reinert, L. K. 2004. An amphibian model to test the effects of xenobiotic chemicals on development of the hematopoietic system. *Environ Toxicol Chem*, **23** (12), 2863–2867.

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