AGRICULTURAL BIOLOGY,
2018, V. 53, № 2, pp. 337-347
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 639.3/.5:574.5:591.1

doi: 10.15389/agrobiology.2018.2.337eng
doi: 10.15389/agrobiology.2018.2.337rus

FUNCTIONAL INDICATORS OF POIKILOTHERMIC AQUATIC SPECIES FROM NATURAL AND ARTIFICIAL WATER BIOCENOSES

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Received May 12, 2016

Abstract

For assessment of sustainability of natural biocenoses and the physiological and immunological state of hydrobionts in aquaculture, it is necessary to know functional parameters of circulating liquids in hydrobionts of different taxonomic groups. The purpose of this study was to analyze the limits of the main indicators of homeostasis in aquatic animals and fish from natural water bodies, i.e. crayfish (*Astacus astacus* and *Pontastacus leptodactylus*), fish (carp Cyprinus caprio L., tench *Tinca tinca* L. and catfish *Silurus glanis* L.), and amphibians (frogs *Rana temporaria* and *Xenopus laevis*). Here, here is the first report on hematologic, cytochemical, biochemical indicators for these species which inhabits natural water bodies or are artificially grown in the conditions of Moscow and Pskov provinces and Chuvashiya region. Hematological investigations included differential count of blood cells of fishes and amphibians in smears stained by the Pappengeim technique; hemolymph of river crayfish was examined in Goryaev chamber. Immunological parameters were evaluated by cytochemical method as an average cytochemical coefficient (CCC) of lysosomal cationic protein in fish blood neutrophils and crayfish haemocytes in the reaction with Bromphenol blue. Biochemical parameters were assessed in blood serum using a biochemical analyzer Chem Well (Awarenes Technology, Inc., USA). The reference constants of homeostasis we found are as follows: the total number of cells in crayfish hemolymph of 700 to 800 per 1 μl; the number of red blood cells in fish of 1-2 million/μl, blood leukocytes in fish of 50-150 thousand per 1 μl. Interspecific differences in haemocyte patterns between *Astacus astacus* and *Pontastacus leptodactylus* were not revealed. Biochemical differences were as follows: glucose concentration in the *Astacus astacus* hemolymph was 64 % higher compared to that in *Pontastacus leptodactylus* whereas the alkaline phosphatase activity was almost 71 % lower. Agranular and semi-agranular haemocytes, along with juvenile forms that we called transparent cells, serve as phagocytes in crayfish. In healthy crayfish, phagocytic activity of these cells, as estimated by the average cytochemical coefficient of the lysosomal cationic protein level, was approximately the same and ranged from 1.5 to 2.0. In fish, we found gender and species-related differences of homeostatic constants. The presence of promyelocytes, the blast forms of leukocytes, in *Tinca tinca* was indicative of more intensive leukopoiesis. The percentage of neutrophils was higher in male *Tinca tinca* due to 2- to 3-fold number of band neutrophils compared to other groups. The level of non-enzyme cationic protein in the lysosomes of neutrophils of female carp, tench and catfish were higher compared to male individuals. The activity of aspartate aminotransferase (ASAT) in male tench and catfish was almost 3 times higher than that in carp. The carbohydrate metabolism in carp and catfish, in terms of lactate concentration, was more than 3 times higher compared to tench. Among the studied amphibians, we observed interspecific and gender differences. The proportion of segmented neutrophils in *Rana temporaria* was more than 4 times higher than that of *Xenopus laevis.* Gender variations in the number of segmented cells were as follows: the cell number in male *Rana temporaria* and *Xenopus laevis* were 27 and 33 % lower than that of females. The blood lymphocyte counts in *Rana temporaria* were significantly lower than that in *Xenopus laevis.* We found gender differences of *Rana temporaria* on biochemical parameters. As compared to the males, the female *Rana temporaria* showed higher activity of ALAT and ASAT (by 6 and 19 %, respectively), creatine kinase (by 29 %), and alkaline phosphatase (by 60 %). The total blood protein content in amphibians was 2-3 %, blood glucose averaged 1-4 mmol/l, triglycerides varied

from 0 to 400 mg%. It is proposed to use parameters of aquatic organisms' homeostasis for ecological monitoring of natural and artificial water biocenoses.

Keywords: natural and artificial water biocenosis, aquatic animals, lower vertebrates, crayfish, *Astacus astacus*, *Pontastacus leptodactylus*, fish, *Cyprinus caprio*, *Tinca tinca*; *Silurus glanis*, amphibians, *Rana temporaria*, *Xenopus laevis*, homeostasis

Growing demand for qualitative products promotes development of aquaculture and artificial reproduction of animal species — traditional members of the essential biocenosis [1]. At the same time, many species become extinct from water biocenosis under the pressure of antropogenous factors which destabilize hydrobiont communities. Thus, crawfish and sturgeons are very rarely found in biocenosis in Moscow Region. In the past years, number of amphibionts had sharply decreased resulting in uncontrolled reproduction of insects — mosquito, fly, and gad-fly. Recently, reduction in the number of amphibiont populations, death of separate populations or species in general becomes more global [2]. It is evident that it is impossible to secure ecologic safety around and inside big cities without artificial regulation of the population number and specific composition of animal communities. Artificial reproduction and use of shellfish, fish, amphibian species, being the biocenesis stability markers, is suppressed by the lack of detailed information on physiological norm for animals, first of all, inhabitants of water reservoirs, the hydrobionts. Since blood is a labile body system, hematological indicators to the most extent reflect physiological properties of such animals and changes of their ecosystem at pollution of water reservoirs, and serve the basis of bioindication method [3].

Invertebrate hydrobionts significantly differ from lower vertebrates not only by body constitution, but also blood system. Breathing pigment in majority crawfish species is hemocianine [4] that makes its color blue. Blood system of shellfish is open: hemolymph circulating in vessels and intercellular cavities consists of liquid part (plasma), and cell components (haemocytes). Many authors highlight three haemocytes in crawfish [5-7]. We have identified four autonomous morphofunctional types of such cells [8] well distinguished at microscoping of freshly collected hemolymph, which were called agranulocytes, semi-agranulocytes, granulocytes, and transparent cells. Agranular haemocytes (GC I) are small (3-17 μm) usually spherical cells with small number of insertions. They remain unchanged at object plate than other types. Poly-granular haemocytes (GC II) are cells of 8-40 μm in size. They are interim cells between two other cell types. They contain small number of different size granules. Their cytoplasm is destructed at object plate and in 30-40 minutes it is hard to distinguish GC II from agranular haemocytes. Granulocytes (GC III) are the biggest hemolymph cells (up to 50 μm and more) with numerous and big granules with high refringency. Outbreak of granules with further dissolution of cytoplasm commences in 15 minutes following collection of hemolymph. Size of transparent cells (GC IV) is nearly 8-35 μm, they are hardly identified and their nucleus is not visible at light microscopy of native hemolymph. Assumedly, they are non-differentiated predecessors of blood cells. Hemolymph in vitro in aerobic context rapidly changes its rheological properties, loses fluidity and transforms into a gel-like mass, haemocytes are exposed within 30-50 minutes to structural and functional changes, gradually transforming from an oval-fusiform cells into round-shaped formations. In anaerobic context, no rheological changes apparently occur in crawfish endolymph (or the process slows down) which is evidenced by continued lymphatic leakage at tamponade of traumatic injury of cuticle (endolymph does not coagulates), whereas gel-form clot is formed in a few seconds upon hemolymph contact with air [9].

Haemocyte functions in crawfish are not studied enough. However, it is

established that different haemocyte types participate in immune protection. Membranes of haemocyte-agranulocyte contain recognizing receptors. Antigen recognition (for instance, β-1,3-glucans fungi or lipopolysaccharides of bacteria Sn) happen at penetration of alien agents followed by activation of the enzyme cascade promoting discharge of phenol oxidase from semi-agranular and granular cells [10]. It is also established that phagocytosis, incapsulation by haemocyte layers, microbial killing, and agglutination of antigens are done by agranular and semi-agranular haemocytes [11]. As apart from the selective immunity in superior vertebrates, crawfish lack genetically transmitted antibodies and tolerance of immune system which provides the basis to assume that hemolymph cells exhibit phagocytosis activity in anaerobic conditions [12, 13]. Crawfish lack hematopoiesis organs, but have blood-forming tissue located at dorsal and dorsolateral surface of ventriculus. There are five types of blood-forming cells, and their number is approximately 1.4×10^6 [14].

Blood cells and immunity factors of hydrobionts are most studied in fish. Cell morphology in fish is highly diversified and is displayed in form, cell sizes, nucleus, granules, specific content of cell elements, and to a significant effect is defined by ecologic conditions of a specie ecosystem. Values of white and red blood cells depend on temperature and pollution of water, hydrochemical mode, content and quantity of consumed feed, stocking density at raising, season, age, and physiologic state [15-17]. As apart from higher vertebrates, blood of lower fishes contains sufficient number of immature cell forms. There is no unique classification of blood cells. That is, some authors [13, 18] divide normoblasts into basophils, polychromatophils, and oxilophils, and classify them as mature cells; the other authors [19] consider normoblasts as immature cells, and divide erythrocytes, by maturity extent, into basophils and polychromatophils. By maturing, cells of erythroid row pass erythroblast, normoblasts, basophilic, polychromatophilic, and mature erythrocyte stages. Subject to commonly accepted terminology, mature leucocytes are divided into granular or grainy (basophils, eosinophils, neutrophils) and agranular or non-grainy cells (lymphocytes and monocytes). Immature forms of lymphocytic row are presented by lymphoblasts, prolymphocytes, monoblasts, and promonocytes. Mieloblasts, promielocytes, mielocytes, and metamielocytes refer to precursors of cells of myeloid (granular) row [20]. As apart from mammals, fish has more hematopoiesis organs. These are gill apparatus and thymus gland stretching from it, lymphatic follicles, gastric mucosa, heart epithelium and vascular endothelium, spleen (in higher vertebrates and bone fishes it serves as blood cell destruction and phagocytosis organ], and kidneys. Hematopoiesis in bone fishes is mostly active in lymphoid organs, kidneys, and spleen, provided that principal blood circulation organ is kidneys (front part]. Formation of erythrocytes, leucocytes, trombocytes, as well as decomposition of erythrocytes occurs in kidneys and spleen. In fish (as apart from mature mammals), mature and young erythrocytes present in peripheral blood do not serve the pathology indicator.

There is scarce information on cellular content of blood in amphibians. It is known that blood erythrocytes in amphibians are bigger than in fishes, mainly nuclear ones [21-24]. Number of erythrocytes in tailed amphibians is approximately 0.07-0.08 million/μl, in untailed, according to various sources, 0.35-0.50 million/μl and 0.38-0.64 million/μl; number of leucocytes is 2.4-21.0 thousand/μl, of trombocytes — 8.5-21.6 thousand/μl [25]. Sometimes, non-nucleated erythrocytes are found (up to 5 %) [19].

For the first time we have studied hematological, cytochemical, and biological indicators in representatives of water fauna in various taxonomic (crustaceans, fish, amphibians) and gender groups in Moscow, Pskov Regions, and Chuvash Republic. The obtained results could be used at assessment of sustainability of

natural biocenosis and the adequacy of keeping conditions of such hydrobionts in aquaculture.

Purpose of present study was to determine benchmark values for principal homeostasis indicators in hydrobionts from artificial and natural water basins.

Techniques. Studies (2005-2015) were conducted on sexually mature and clinically healthy animals of different taxonomic groups: two species of crawfish: *Astacus astacus* and *Pontastacus leptodactylus*, three fish species (*Cyprinus caprio* L., *Tinca tinca* L., *Silurus glanis* L.) and two amphibian species (*Rana temporaria* and *Xenopus laevis*) from the natural and agricultural water basins of the temperate zone of the European Russia (except for *Xenopus laevis* raised in aquarium conditions). *Astacus astacus* inhabits basins of Pskov Region, *Pontastacus leptodactylus* — of Moscow Region. Carp and catfish were raised in fishing ponds of fish-breeding farm Kirya (Chuvash Republic), tench — in fish-breeding farm Osenka (Moscow Region). Animal groups were formed based on analogue principle, accounting for the generic inhering, sex, age, and live mass. Number of groups depended on availability of object and varied within the limits of small sample (from 5 to 20 imdiciduals).

Subject to our developed methodology [26], probes of circulating liquids of crawfish and fish were aseptically collected by noninvasive method. Hemolympth of crawfish was collected in vivo by puncture of ventral sinus, fish blood from the tail vein. In amphibians, blood for preparation of wipes for hematologic and cytochemical studies were collected in vivo from finger and for biochemical analysis from the heart.

Upon Pappenheim staining method which allows discrimination of nucleus and cytoplasmatic inclusions, cells were first subjected to fixation by May-Gruenwald solution for 3 minutes. After the reagent was removed by washing with distilled water, the preparations were treated with Romanovsky solution during 40 minutes, washed by tapped water, and air dried.

Total number of haemocytes (TNH) for calculation of haemocyte formula in crawfish was counted in Goryaev's chamber in native hemolymph just after collection. For determination of erythropoiesis indicators and differential leucocytes count in fish (leucocyte formula), peripheral blood wipes stained by Pappenheim were used. Hematopoiesis activity in fish was assessed as the portion of immature erythrocyte forms. Amphibian blood cells were counted in Goryaev's chamber. For erythrocyte count, blood sample was diluted 200 times with 0.9 % NaCl solution (20 μl of blood and 4 ml of the solution). Count of erythrocytes X_e in 1 μl of blood was calculated as $X_e = (a_e \times 4000 \times 200)/80$, where a_e is the number of cells in 80 small squares of Goryaev's chamber. For leucocyte count, sample was diluted 20 times with 5 % solution of acetic acid with methylene blue. Number of blood leucocytes X_l per μl was calculated as $X_l = (a_l \times 250 \times 20)/100$, where a_l is the number of leucocytes in 100 large squares of Goryaev's chamber. Digital microscope Optika DM 15 with software OPMIAS (OPTIKA Micro Image Analysis Software) (PriborUfa LLC, Russia) was used.

Immunological indicators were cytochemically assessed by mean cytochemical coefficient (CCC) of lysosomal cationic protein in blood neutrophils of fish and haemocytes of crawfish in test with bromophenol blue [27] adapted for hydrobions [28]. CCC for fish and crawfish was calculated:

$$CCC = [0 \times N_0(H_0) + 1 \times N_1(H_1) + 2 \times N_2(H_2) + 3 \times N_3(H_3)]/100,$$

where $N_0(H_0)$, $N_1(H_1)$, $N_2(H_2)$, $N_3(H_3)$ (%) are the number of neutrophils in fish (haemocytes in crawfish) with activity of 0, 1, 2, and 3 points.

During biochemical studies, hemolymph of crawfish was centrifuged for 5 minutes at 3000 rpm and 6 °C to prevent rapid coagulation. For production of serum, fish blood was placed in dry sterile vial and allowed for 1 hour at room

temperature, afterwards serum was carefully collected using syringe with thin needle, frozen at –15...–20 °C and transported in frozen state in thermal containers with ice to the laboratory. Biochemical blood indicators in fish and hemolymph indicators in crawfish were determined in a programmed automated analyzer Chem Well (Awareness Technology, Inc., USA), with the use of reagent toolkits of JSC Vital Development Corporation (Saint Petersburg) (analysis of proteins by biuret test method) subject to the producer protocol.

Mean values (M) and standard errors of the mean (±SEM) were calculated. Results were processed by variation statistical methods by Studen's t-test. Deviations were statistically significant at $P < 0.05$.

Results. We tested hematologic, cytochemical, and biochemical indicators, based on which conclusion on physiological norm for representatives of species was drawn.

Specific properties of homeostasis in crawfish. Crawfish is a group of cultivated hydrobionts, physiologic properties of which is the least studied. General clinical analysis of internal environment in crawfish provides the objective information on animal adaptiveness to ecosystem conditions and may be used for biomonitoring of environment in general and water environment in particular. Characteristics of hematologic, biochemical, and cytochemical indicators in both species of crawfish are illustrated in Table 1.

1. Hemolymph characteristics in two crawfish species from natural aquacenosis (M±SEM, Moscow and Pskov regions, 2010-2012)

Indicator	*Astacus astacus* (a) (n =10)	*Pontastacus leptodactylus* (n =10)
Haemocyte formula, %:		
agranular cells	40.0±3.9	34.9±4.8
semi-agranular cells	24.2±5.7	29.7±3.4
granular cells	27.8±2.8	32.1±2.4
transparent cells	8.0±1.9	3.3±1.6
Hematologic and biochemical indicators:		
TNH, kiloliter/μl	384±111	911±137[a]
glucose, μmol/l	2.2±0.6	< 0.5[a]
ALAT, IU/l	80.6±11.7	55.1±17.8
ASAT, IU/l	57.7±7.3	55.3±33.5
ALP, IU/l	17.1±2.1	78.0±20.2[a]
Cytochemical indicators:		
CCC, units	1.70±0.06	1.87±0.17

Note. TNH — total number of haemocytes, ALAT and ASAT — alanine and aspartate aminotransferases, respectively, ALP — alkaline phosphatase, CCC — mean cytochemical coefficient.

[a] Differences from *Astacus astacus* are statistically significant at $P < 0.05$.

In endolympth, the counts of all three cell types (agranular, semi-agranular, granular) varied in average within 32-35 %. However, in *P. leptodactylus* number of transparent cells was by 18.2 % less than in *A. astacus*. For other elements, interspecies differences were insignificant. Mean cytochemical coefficient of lysosomal cationic protein in haemocytes of crawfish *P. leptodactylus* is 9.4 % higher than in *A. astacus*.

This experiments show several trends for biochemical indicators of endolympth in crawfish. Activity of alanine aminotransferase of hemolympth (ALAT) in *A. astacus* was trice higher than in *P. leptodactylus*. Other substrate blood indicators change more notably. Mean glucose concentration in hemolympth in *A. astacus* exceeded that in *P. leptodactylus* by 64 %, while activity of alkaline phosphatase (ALP) was about 5 times lower.

Agranular and semi-agranular haemocytes are phagocytes in crawfish. Besides, so called transparent cells have phagocytosis ability (assumedly, juvenile forms) [9]. Our experiments show that phagocyte reserve in two crawfish species is approximately similar.

Homeostasis in fishes. In evolutionary hierarchy, fishes are lower than warm blooded animals and, accordingly, change limits of indicators of the internal environment in vivo are wider in them. In case of carp, tench, and catfish (Table 2), hematopoiesis occurs in approximately similar manner. Leucopoiesis is more intensive in tench (promieocytes, the blast leucocyte forms are

present). Due to rod nuclear cells, the amount of neutrophils is 2-3 times higher in female tench than in other groups. No eosinophils were found in all fish species, basophils were found in female tench, catfish, and also in female carp at insignificant level.

2. Hematologic indicators in fish species (*M*±SEM, fish breeding farms, Volgograd Region, Chuvash Republic, 2010-2012)

Indicator	carp *Cyprinus caprio* L.		Tench *Tinca tinca* L.		Catfish *Silurus glanis* L.	
	males (a) (n =23)	females (b) (n =10)	males (c) (n =7)	females (d) (n =5)	males (e) (n =12)	females (f) (n =10)
Erythropoiesis, %						
Hemocytoblasts, Erythroblasts	0.3±0 2	0.6±0.2	1.0±0.4	–	0.7±0.4	–
Normoblasts	2 9±0 4	3.4±0.3	2.9±0.1	3.0±0.1	2.7±0.4	3.0±1.4
Basophiluıc erythrocytes	8 6±0 4	9.1±1.1	6.0±3.8	12.1±4.2	11.6±4.0	7.5±0.7
Mature erythrocytes	88 2±1 5	86.9±1.4	90.1±1.1	84.9±4.3	85.0±4.4	89.5±2.1
Leucocyte formula, %						
Mieloblasts	–	–	–	–	–	–
Promielocytes	–	–	1.0±0.7	2.0±1.4	–	–
Mielocytes	0 8±0 4	1.3±0.5	–	5.7±1.7 [a,b]	0.5±0.4[d]	1.0±0.4d
Metamielocytes	4 0±0.9	4.3±0.4	–	6.2±4.2	3.0±1.4	3.5±0.7
Neutrophils:						
rod nuclear	1 4±0 3	1.0±0.4	6.0±0.2[a,b]	2.0±0.9[c]	0.7±0.5[c]	1.5±0.4[c]
microxyphil nuclear	1 6±0 4	2.4±0.5	3.2±0.4[a]	4.5±0.1[a,,b]	4.3±0.6[a,b]	4.5±0.8[a,b]
total	3 0±0 3	3.4±0.9	9.2±0.9[a,b]	6.5±0.9[a,,b]	5.0±0.8[a]	6.0±1.2[a]
Eosinophils	–	–	–	–	–	–
Basophils	0 4±0 2	0.1±0.2	2.3±0.8 [a,b]	–	0.3±0.3 [c]	–
Monocytes	3 0±0 3	2.2±0.5	2.1±1.1	5.5±3.5	3.3±2.0	2.5±0.7
Lymphocytes	88 8±1 2	88.7±1.3	85.4±4.4	74.1±5.6[a]	87.9±2.3[d]	87.0±2.8
Phagocyte activity						
CCC, units	1 81±0 07	1.94±0.05	1.68±0.01[b]	2.05±0.01[c]	1.30±0.15[a,b,c,d]	1.72±0.11[e,f]
Biochemical values						
ALAT, IU/l	40 2±10 5	41.3±12.2	39.6±8.9	32.6±5.9	45.0±4.4	75.1±12.8
ASAT, IU/l	164±13	133±39	346±18[a,b]	310±40[a,b]	402±12[a,b]	367±29[a,b]
Glucose, μmol/l	3 6±1 2	4.5±1.1	9.4±1.3[a]	6.6±0.5[a]	7.4±1.1[a]	8.1±1.3[a]
Creatine kinase, IU/l	3896±63	3877±161	3054±18 [a,b]	2990±107 [a,b]	527±93[a,b,c,d]	1185±430[a,b,c,d]
Lactate, mg/dl	66 9±7 5	68.5±5.7	19.9±4.5 [a,b]	19.1±2.7 [a,b]	116.2±5.3[a,b,c,d]	121.1±9.8[a,b,c,d]
ALP, IU/l	25 5±1 5	17.5±0.5[a]	43.6±4.7 [a,b]	56.3±11.4 [a,b]	9.9±6.3[a,c,d]	9.3±4.0[a,c,d]
Albumin, g/dl	11.5±3.4	9.1±1.7	15.2±1.7	14.8±1.4	12.2±0.3	13.7±2.7
Total protein, g/l	26.8±6.4	22.3±1.7	24.9±3.3	21.5±1.2	29.9±2.5	31.0±5.1
Triglicerides, mg/dl	124±42	105±32	76±33	94±25	271±105	178±25
Cholesterol, mg/dl	109±12	118±21	121±39	133±16	134±28	107±26

Note. ALAT and ASAT — alanine and aspartate aminotransferase, ALP — alkaline phosphatase, CCC — mean cytochemical coefficient. Dash means no available data.
[a,b,c,d,e,f] Letters in upper index indicate the variant differences with which are statistically significant at $P < 0.05$.

Quantity of non-enzyme cationic protein in neutrophil lysosomes (CCC) in females was higher than in males; in tench and catfish the differences are reliable. Differences could be explained by strengthening of non-specific cell immunity in females. Activity of aspartate aminotransferase (ASAT) in male tench and catfish was approximately 3 times higher compared to carp, with high reliability, i.e. for carp and tench $t = 13.5$. Biological role of ASAT is transamination important for energy metabolism. Any states requiring urgent mobilization of protein components to cover energy needs of a body are associated with adaptive hormonally stimulated biosynthesis of ASAT. Obtained results evidence on higher stress tolerance in tench and catfish as compared to carp. Reliable over 3-fold growth of lactate content in carp and catfish compared to tench is testified, which evidences on intensive carbohydrate metabolism. At the same time, mineral metabolism, according to ALP activity, in male tench was 2-3 times more intensive.

In general, the studied clinically healthy fishes showed differences in leukogram: tench had higher level of macrophages (neutrophils) that indicates phagocytosis potential. Phagocytic activity of these cells in studied female fishes was somewhat higher than in males.

Homeostasis in amphibians (*Rana temporaria*, *Xenopus laevis*).

In scientific literature, homeostasis indicators in amphibians are discussed unreasonably rarely. In our studies of *R. temporaria* the erythrocyte count ranges within 0.12-0.37 million/μl in males, and 0.22-0.39 million/μl in females; leucocytes range within 0.14-0.38 million/μl in males, and 0.13-0.47 million/μl in females. Frogs show both gender and interspecific differences in leucocyte formula (Table 3).

3. Hematological indicators in frog species (*M*±SEM)

Indicator	*Rana temporaria*		*Xenopus laevis*	
	males (a) (n =10)	females (b) (n =10)	males (n =5)	females (n =5)
Erythropoiesis, %:				
hemocytoblasts, erythroblasts	0.8±0.3	0.4±0.4	1.5±2.1	2.1±0.5
normoblasts	2.8±0.6	2.4±0.5	5.0±2.8	0.9±0.3
mature erythrocytes	96.4±0.7	97.2±0.9	93.5±1.3	97.0±0.6
Leucocyte formula, %:				
metamielocytes	0.2±0.3	–	–	0.8±0.4
rod nuclear neutrophils	0.2±0.3	0.6±0.4	–	1.2±0.6[a]
microxyphil nuclear neutrophils	12.2±0.9	16.8±1.5[a]	2.7±0.5 [a,b]	4.3±0.3 [a,b]
total neutrophils	12.4±0.9	17.4±1.5[a]	2.7±0.5 [a,b]	5.5±0.4 [a,b]
eosinophils	3.0±0.7	3.2±0.9	1.8±0.4	0.9±0.4 [a,b]
basophils	–	0.4±0.4	0.5±0.7	–
monocytes	2.8±0.5	2.0±0.4	2.6±0.8	2.2±0.5
lymphocytes	81.6±1.0	77.0±1.7	92.4±0.8 [a,b]	90.6±1.2 [a,b]
Bacterial activity, units	1.78±0.27	1.78±0.24	2.02±0.06	1.74±1.20

N o t e. BA — bactericide activity of blood neuthrophils. *Rana temporaria* frogs were taken from the natural aquacenosis (Moscow Region, 2014), *Xenopus laevis* grew in an aquarium. Dash means that indicators falls outside the device sensitivity limits.

[a,b] Letters in upper index indicate the variant differences with which are statistically significant at $P < 0.05$.

Blood of *Rana temporaria* males and *X. laevis* females contain metamielocytes. The number of rod nuclear neutrophils was 67 % higher in *X. laevis* females compared to males. Microxyphil nuclear neutrophils in *R. temporara* were 4-fold as much as in *X. laevis*. We revealed gender variations in microxyphil nuclear cells which counts were 27 and 33 % less in *R. temporara* and *X. laevis* males than in females. Eozinophils of white blood granulocyte row in males and females of *R. temporara* was comparable in number, and reliably lower in females of *X. laevis*. Number of blood lymphocytes in *R. temporaria* was lower than in *X. laevis*. The value of neutrophil CCC in males and females was about the same in *Rana temporara* and 22 % higher in males of *Xenopus laevis* compared to females.

4. Biochemical blood indicators in males and females of *Rana temporaria* from natural aquacenosis (*M*±SEM, Moscow Region, 2014)

Indicators	Males (a) (n =10)	Females (n =10)
ALAT, IU/l	164±36	174±33
ASAT, IU/l	88±29	109±36
Glucose, μmol/l	0.8±0.3	1.4±0.2
CK, IU/l	985±159	1388±362
Creatine, μmol/l	46±10	47±5
LDH, IU/l	3798±417	3430±220
Lactat, mg/dl	49±16	52±6
Urine acid, μmol/l	262±110	377±151
ALP, IU/l	28±16	69±45
Albumin, g/dl	21±2	25±0.5
Urea, mg/dl	48±2	56±1[a]
Total protein, g/l	24±6	34±2
Trigliceride, mg/dl	3±1	11±6
Cholesterol, mg/dl	57±11	85±18
Hemoglobin, g/l	93±15	172±6[a]

N o t e. ALAT and ASAT — alanine and aspartate aminotransferases, CK – creatinine kinase, LDH — lactate dehydrogenase, ALP — alkaline phosphatase.

[a] Letter in upper index indicates the variant differences with which are statistically significant at $P < 0.05$.

Total blood volume in amphibians is small that challenges biochemical testing. That is why we managed to determine only few indicators (Table 4). Sexual differences were characteristic of *R. temporaria*. In females as compared to males ALAT and ASAT activity is 6 and 19 % higher, creatine kinase is 29 % higher, and alkaline phosphatase is 60 % higher.

Glucose ensures metabolic processes in vivo and is important in energy metabolism in animals. In *R. temporaria* females, as compared to other studies species, this indicator exceeded that in males to a greater extent (by 43 %). Possibly, nearly 2-

fold excessive content of glucose in females is caused by higher dependence of females from physical environmental factors, especially during reproduction, which makes such indicator the most important one. Interestingly, amphibian females are also superior to males in other biochemical blood parameters (e.g. content of creatine, lactate, urine acid, albumin, urea, and total protein is 30 % higher).

Gender specificity of blood lipid metabolism in amphibians of the studied specie manifests itself in superiority of females over males (blood triglycerides and cholesterol are 68 % and 33 % higher, respectively). Other assessed biochemical blood indicators, except for activity of lactate dehydrogenase, were higher in *R. temporara* females than in males.

It should be noted that blood microxyphil nuclear cells of *R. temporara* males and females (see Table 3) exceeded in number those of *X. laevis* by 78 and 76 %, the number of neutrophils was 78 and 71 % more, and the count of eosinophils was 40 and 69 % more. Number of blood erythroblasts in *X. laevis* males and females as compared to *R. temporara* was 47 and 80 % higher. Normablasts in *R. temporara* males was 44 % lower than in *X. laevis* male. *R. temporara* females left behind *X. laevis* females as per rod nuclear and microxyphil nuclear neutrophils, their total amount and number of eosinophils (by 67, 76, 71, and 69 %, respectively). Number of rod nuclear neutrophils in *X. laevis* males exceeded the indicator of *R. temporaria* by 80 %. Portion of eosinophils in males and females of *R. temporaria* was 44 and 67 % higher compared to *X. laevis.*

Interspecific differences between studied amphibians in number of lymphocytes are not so evident. By the content of agranular haemocytes, *X. laevis* males were somewhat superior to *R. temporaria* males. The trend for lymphocytes in males and females was similar. However, average number of lymphocytes in *R. temporaria* is somewhat higher (by 15 %) compared to *X. laevis.* CCC values in *R. temporaria* and *X. laevis* differ insignificantly.

Therefore, there are specific and gender differences of the studies amphibians by homeostasis indicators that reflects adaptation in various biotopes.

It should be noted that crustaceans, fish, and amphibians have a number of common homeostatic traits, regardless of the great evolutionary remoteness. Circulating liquids contain granular, agranular, and by juvenile cell forms. In haemocyte formula of various crawfish species we identified four haemocyte types in close percentage relationship. Leucogram in the studied lower vertebral hydrobionts is similar though differs in several types of leucocytes, and intensity of erythropoiesis is similar. Biochemistry of internal environment in such different taxones is also sufficiently similar. We detected glucose, proteins, triglycerides in comparable quantities in hemolympth of crawfish, as well as blood plasma in fish and amphibians.

Thus, we suggest the criteria to estimate the adaptiveness of hydrobionts, sustainability of natural biocenoses and/or adequacy of aquaculture conditions. These parameters are as follows: total hemolymph cell number within 700-800 per μl for crawfish, the erythrocyte count of 1-2 million per μl and leucocyte count of 50-150 thousand per μl in fish and frogs. In vertebrate hydrobionts, the blood total protein concentration is 2-3%, the glucose concentration is 1-4 μmol/l and the concentration of triglycerides is 0-400 mg%. Mean cytochemical coefficient of non-enzyme cationic protein of phagocytic cell lysosomes is 1.5-2.1.

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