# ECOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE LEUKOCYTE PROFILE AND HUMORAL IMMUNITY OF *PELOPHYLAX RIDIBUNDUS* (ANURA: RANIDAE) IN THE LOWER VOLGA REGION UNDER HEMOPARASITIC INVASION

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**Abstract.** The molecular genetic structure of populations and an assessment of adaptation mechanisms were performed for the marsh frog (*Pelophylax ridibundus*) inhabiting water bodies differing in the type of environmental stress, defined by the level of environmental pollution (classified as water quality classes 4 and 5). Molecular genetic diagnostics revealed the presence of individuals with introgressive mtDNA of the Anatolian water frog (*P.* cf. *bedriagae*) in the marsh frog sample. Species identification was carried out using a multiplex PCR test system. We detected haplotypes and alleles of the invasive Anatolian marsh frog (*Pelophylax* cf. *bedriagae*) in marsh frog populations. The samples revealed a high infection intensity with intracellular parasites of the class Conoidasida (representatives of the genus *Dactylosoma* from the family Dactylosomatidae, and species of the genus *Hepatozoon* from the family Hepatozoidae) and extracellular parasites (class Nematoda, family Onchocercidae, microfilariae larvae). The dominant hemoparasite was *Dactylosoma* sp., while microfilariae larvae were extremely rare; representatives of the genera *Hepatozoon*, *Trypanosoma*, and *Lankesterella* showed moderate prevalence. It was found that under extreme environmental pollution (class 5), inhibition of humoral responses occurs (a decrease in the level of large and an increase in the level of small immune complexes), which is interpreted as a compensatory shift of resources towards cellular defense mechanisms in the marsh frog. Against the background of environmental stress, the presence of intraerythrocytic parasites acts as an additional modulator of amphibian cellular immunity.

**Keywords:** *Pelophylax ridibundus*, environmental stress, hemoparasites, immune complexes, blood cell profile, leukocyte indices.

## Introduction

Amphibians play an important role in the global ecosystem and serve as classic bioindicators, are currently experiencing a sharp decline in populations worldwide (Kiesecker *et al.*, 2001; Blaustein *et al.*, 2010; Luedtke *et al.*, 2023). Current research reveals that this decline is compounded by habitat degradation, climate change, and infection by viruses, fungi, and parasites (Scheele *et al.*, 2019; Netherlands *et al.*, 2020b).

A key ecological problem concerns the population-level adaptive responses of the immuno-hematological system in natural amphibian populations to changing climatic (Raffel *et al.*, 2006) and hydrochemical environmental conditions (Gomes *et al.*, 2019), despite the fact that the amphibian immune system is evolutionarily adapted to rhythmic seasonal climate changes (Nelson & Demas, 1996). The combined effect of these factors, leading to homeostasis disruption, remains largely unexplored. However, climate change and hydrochemical pollution can increase the susceptibility of amphibians to parasitic infections by inducing immunosuppression (Blaustein *et al.*, 2012; Rubenina *et al.*, 2021). Several studies have emphasized the relevance of investigating population-ecological aspects of amphibian immune responses under conditions of increasing anthropogenic pressure (Daszak *et al.*, 2000; Lips *et al.*, 2006). It is noted that forest frag-

mentation and changes in the hydrological regime directly correlate with an increase in the infection intensity of amphibians by hemoparasites (Ferreira *et al.*, 2020). The physiological features of the immune system provide amphibians with high protection against stress, and blood parameters represent a valuable assessment of the organisms resistance and reactivity to a complex of environmental factors (Davies *et al.*, 2008), including parasitic infections that significantly impact the ectothermic animal populations health status (Blaustein *et al.*, 2003).

Thus, the aim of this work was to assess qualitative and quantitative differences in the immune function of the marsh frog, *Pelophylax ridibundus*, infected with hemoparasites and inhabiting urban water bodies with varying levels of environmental stress driven by pollution.

**Materials and Methods**

The study used samples from natural populations of *P. ridibundus* collected using dip nets in two water bodies of the Astrakhan region on August 27, 2025 (44 individuals, 28 males and 14 females).

The sample 1 ($n$ = 22, 11 males and 11 females) collected using dip nets in the coastal zone of the Akhtuba River (near the village of Komsomolskiy, Krasnoyarsk District, Astrakhan Oblast; 46.848° N, 47.926° E) on August 27, 2025. The banks are low, swampy, with dense reed thickets. The river current is weak, and the substrate consists of sand and clay. The proximity of human settlements results in a high intensity of anthropogenic impact.

The sample 2 ($n$ = 22: 19 males and 3 females) collected using dip nets in the coastal zone of the Bereket River (near the village of Stepnoy, Krasnoyarsk District, Astrakhan Oblast; 46.672° N, 48.237° E). The water body is flowing, with a weak current. The water level rises significantly during floods. The substrate consists of sand and clay.

Molecular genetic analysis was performed for 16 individuals (6 from sample 2 and 10 from sample 10). DNA was extracted from blood samples or distal toe phalanges preserved in 70% ethanol. DNA isolation was performed using the Biolabmix DU250 kit and the salt extraction method (Aljanabi & Martinez, 1997) combined with proteinase K lysis. The mitochondrial cytochrome *c* oxidase subunit I (COI) gene and the nuclear first intron of the serum albumin (SAI-1) gene were selected, for which a multiplex PCR assay had been developed (Ermakov *et al.*, 2019). Species-specific primers for amplifying fragments of different lengths were taken from the same study. PCR mixtures for mtDNA and nuclear DNA were prepared separately, with equal primer concentrations in each reaction. PCR amplification was carried out under the following conditions: initial denaturation at 94 °C for 30 sec, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C (for SAI-1) and 62 °C (for COI) for 30 sec, and extension at 72 °C for 30 sec. Each 12 μL PCR reaction mixture contained 30–50 ng of frog DNA, 0.25 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.2 μL of 10× PCR buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KCl), and 1 unit of Taq polymerase (Thermo Scientific). PCR products were visualized by electrophoresis in 1.5% agarose gel stained with ethidium bromide. Gels were run for 30 min at 100 V in 1× TAE buffer. A 1,000+ bp DNA ladder (Evrogen, Russia) was used for fragment length estimation.

Water samples were collected simultaneously with frog sampling for hydrochemical analysis. A Hach DR-2800 spectrophotometer (HACH LANGE GmbH, Germany) was used to measure concentrations (mg/L) of the pollutants (iron, manganese, copper, nickel, zinc, lead, chromium, cobalt, nitrates, nitrites, sulfates, and sulfides). The Specific Combinatorial Water Pollution Index (SCWPI) was calculated according to RD 52.24.643-2002, following optional list No. 3 (parameters relevant for specific research studies). Only parameters exceeding the maximum permissible concentrations for fisheries water bodies (MPC) were included in the calculations (Order of the Ministry of Agriculture of Russia No. 552, 2016).

Body mass was measured using Highland series electronic laboratory scales (HCB 123, ADAM Equipment Co., Ltd), with a permissible error limit of ±0.01 g. Blood was collected

from the heart using a 1 mL insulin syringe with a fixed needle. Thin blood smears were prepared, air-dried, fixed in absolute ethanol, and stained with Giemsa stain for 20 min (Menshikov *et al.*, 1987). Identification (Telford, 2009) and enumeration of intraerythrocytic parasites were performed on 500 erythrocytes using a Meiji Techno MT 4000 series light microscope (Japan) with oil immersion (×1500 magnification).

The following infection parameters were calculated: prevalence (P, %) – the proportion of individuals infected with hemoparasites relative to the total number of individuals examined; mean intensity of infection (I, relative units) – the average number of parasites per infected individual; and abundance (A, relative units) – the average number of hemoparasites per individual examined.

Leukocyte differential counts were determined from stained smears, and the following integral leukocyte indices were calculated: blood cell index; neutrophil reactive response; lymphocyte–granulocyte index; neutrophil–lymphocyte ratio; lymphocyte–eosinophil ratio; neutrophil–eosinophil ratio; leukocyte shift index; neutrophil nuclear shift index; and leukocyte intoxication index.

Serum levels of circulating immune complexes (optical density units per 10 mL serum) were assessed by selective precipitation in polyethylene glycol (PEG) solution using an SF-2000 spectrophotometer (OKB Spektr LLC, Russia) at wavelengths of 200, 280, 350, and 450 nm (Grinevich & Alferov, 1981; Khokhlova *et al.*, 2004). Large (PEG, 3.75%) and small (PEG, 7.0%) immune complexes were determined, and their ratio (aggregation index) was calculated.

Given the distribution patterns, central tendencies and dispersion of the studied parameters were described using the median (Me) and interquartile range (IQR). Data analysis was performed using nonparametric tests: Mann-Whitney U test (for pairwise comparisons), Kruskal-Wallis test (for multiple group comparisons by a single factor), Dunn's test (for multiple pairwise comparisons), and z-test (for proportion comparisons). The statistical significance level was set at $\alpha = 0.05$. All calculations were performed using Statistica 8 software (StatSoft Inc., OK, USA).

## Results

*Molecular detection*

Molecular identification revealed a high proportion of alleles and haplotypes of the Anatolian marsh frog (*P.* cf. *bedriagae*) in marsh frog populations. Mitochondrial DNA of *P.* cf. *bedriagae* predominated over that of the European marsh frog, *P. ridibundus*, (62.5%), while nuclear alleles were present in 12.5% of individuals. The genotype distribution in the population was as follows (mtDNA haplotype / nuDNA alleles): B/RR – 56.3%, R/RR – 31.3%, B/RB – 6.3%, and R/RB – 6.3%. The dominant genotype was that of the European marsh frog with the Anatolian marsh frog mitochondrion.

*Characteristics of abiotic habitat conditions of marsh frogs*

The water bodies in the Astrakhan region where marsh frogs were collected were characterized by high levels of water pollution. Based on hydrochemical parameters, the water of the first site corresponded to quality class 5 (SCWPI = 15.69, extremely polluted), with exceedances of fishery MPC values for manganese (160× MPC), copper (380× MPC), sulfides (68× MPC), zinc (11× MPC), cobalt (13.5× MPC), chromium (4.7× MPC), iron (4.4× MPC), and nickel (2.1× MPC). The second water body (SCWPI = 6.64) corresponded to quality class 4, category B (polluted), with exceedances for copper (80× MPC), manganese (20× MPC), sulfides (20× MPC), zinc (8× MPC), chromium (2.9× MPC), sulfates (0.7× MPC), nickel, cobalt, and iron (0.3× MPC), and nitrites (0.2× MPC) (Table 1).

*Hemoparasite identification and prevalence*

High infection rates with intracellular parasites of the class Conoidasida (species of the genus *Dactylosoma* from the family Dactylosomatidae, and species of the genus *Hepatozoon* from the family Hepatozoidae) and extracellular parasites (class Nematoda, family Onchocercidae, microfilariae larvae) were detected in both samples.

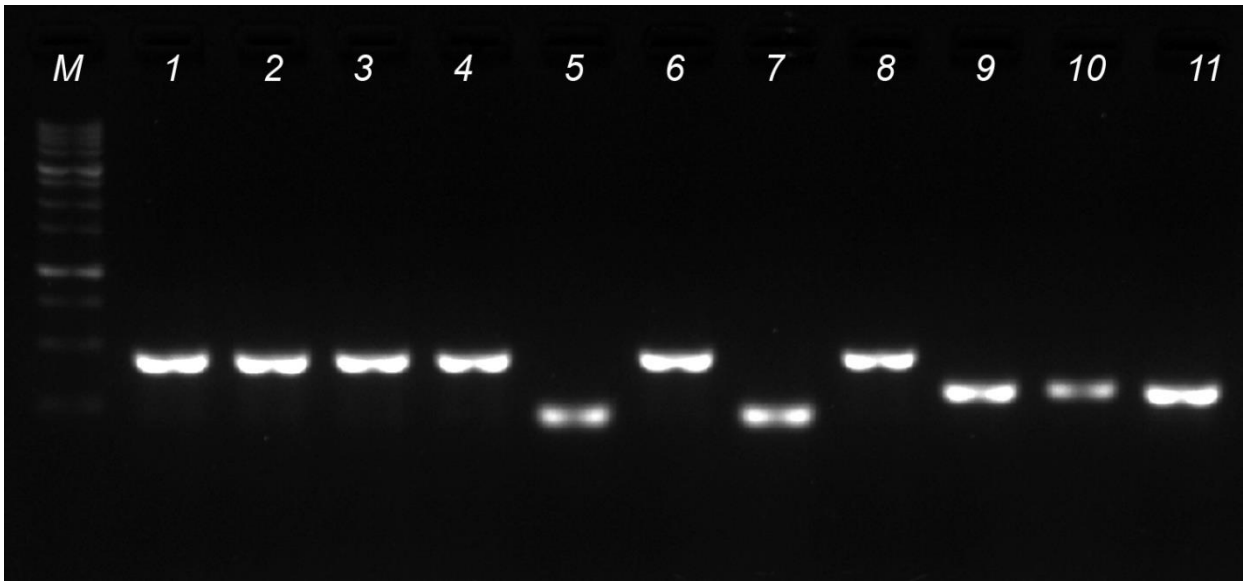



**Fig. 1.** Electrophoresis results of multiplex PCR products of the COI gene fragments of *Pelophylax ridibundus* from the Astrakhan region: M – DNA length marker (1,000 bp); 1-4, 6, 8 – *P.* cf. *bedriagae*; 5, 7 – *P. ridibundus*; 9-11 – *P. lessonae* from Mari El (control)

*Table 1*

**Water chemistry analysis of the studied aquatic environments**

| Samples | Pollution parameters, concentration (mg/L) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fe | Mn | Cu | Cr | $NO^3$ | $NO^2$ | $SO^4$ | S | Zn | N | Co |
| Sample 1 | 0.44 | 1.60 | 0.38 | 0.09 | 0.80 | 0.03 | 103 | 0.34 | 0.11 | 0.02 | 0.14 |
| Sample 2 | 0.03 | 0.20 | 0.08 | 0.06 | 0.80 | 0.01 | 65 | 0.10 | 0.08 | 0.003 | 0.003 |

*Table 2*

**Prevalence of blood parasites (%) in two populations of the marsh frog (*Pelophylax ridibundus*) from Astrakhan Oblast**

| Samples | *Dactylosoma* | *Hepatozoon* | *Lankesterella* | *Trypanosoma* | Microfilariae larvae (%) |
|---|---|---|---|---|---|
| Sample 1 | 68.0 | 50.0 | 36.0 | 18.0 | – |
| Sample 2 | 68.0 | 45.0 | 18.0 | 27.0 | 4.5 |

Of the 44 *P. ridibundus* individuals examined, hemoparasites were detected in 39 specimens. In the first sample, *Dactylosoma* sp. was found in 15 individuals (68%), *Hepatozoon* sp. occurred in 11 individuals (50%), *Lankesterella* sp. occurred in 8 individuals (36%), *Trypanosoma* sp. was found in four infected individuals (18%); microfilariae larvae were not detected. In the second sample, the situation was similar. *Dactylosoma* sp. was found in 15 individuals (68%), *Hepatozoon* sp. occurred in 10 individuals (45%), *Lankesterella* sp. occurred in four individuals (18%), *Trypanosoma* sp. was found in six infected individuals (27%), and microfilariae larvae were detected in one individual (4.5%) (Table 2).

The overall patterns of total invasion differed substantially between males and females. When comparing the proportion of infected cells between the two samples, individuals from the first sample exhibited a significantly higher proportion of infected cells ($z = 2.88$, $p = 0.004$), as well as higher prevalence (90.9%), mean intensity of infection (5.7 relative units), and abundance (5.2 relative units) compared to the second sample.

A significant difference in the proportion of infected cells was found between females ($z$ = = 3.33, $p$ = 0.001), in contrast to males from both samples ($z$ = 1.05, $p$ = 0.290). Males were more susceptible to infection both under conditions of elevated pollution (water quality class 5) ($z$ = 2.4, $p$ = 0.016), as confirmed by higher abundance (6.3 relative units) and mean intensity of infection (6.9 relative units), and under conditions of reduced hydrochemical stress (water quality class 4) ($z$ = 4.47, $p$ < < 0.001). At the same time, males in the second sample differed from females by lower prevalence (84.2%), mean intensity of infection (3.6 relative units), and abundance (3.1 relative units) (Table 3).

*Immune complex levels*

The functional activity of the immune response in infected and uninfected marsh frogs, assessed by circulating large ($U$ = 0.70, $p$ = = 0.48) and small ($U$ = 0.11, $p$ = 0.91) immune complex levels, did not differ significantly. However, substantial sex-related differences were found in infected individuals inhabiting water bodies with different types of environmental stress determined by pollution levels.

The highest level of small immune complexes was observed in infected males from the first sample (86.9/36.3), which significantly differed from males ($D$ = 3.83, $p$ = 0.0007) and females ($D$ = 3.27, $p$ = 0.006) of the second sample. In infected male marsh frogs, unlike females inhabiting more favorable hydrochemical conditions, the level of large immune complexes in serum was statistically significantly higher compared to infected females ($D$ = 3.12, $p$ = 0.01) and was comparable to the level of males and females inhabiting the extremely polluted environment.

The elevated mean aggregation index in males (water quality class 4) exceeded that of males ($D$ = 5.49, $p$ < 0.001) and females ($D$ = = 3.59, $p$ = 0.001) from the extremely polluted environment, whose aggregation index values were 2.43 and 1.66 times lower, respectively (Table 4).

*Blood leukocyte profile*

Differences in hematological parameters were revealed between uninfected and infected individuals inhabiting the studied water bodies. First, infected marsh frogs showed increased peripheral blood counts of leukocytes ($U$ = = 2.37, $p$ = 0.01) and erythrocytes ($U$ = 2.04, $p$ = 0.04) compared to uninfected individuals. Infected individuals were characterized by an increased proportion of eosinophils ($U$ = 1.96, $p$ = 0.04) and elevated blood cell index ($U$ = 2.08, $p$ = 0.03) and leukocyte shift index ($U$ = 2.67, $p$ = 0.007). The peripheral blood lymphocyte count ($U$ = 3.10, $p$ = 0.001), as well as the lymphocyte-eosinophil ratio ($U$ = 3.10, $p$ = = 0.001) and lymphocyte-granulocyte index ($U$ = 2.29, $p$ = 0.02), were decreased in infected individuals compared to uninfected ones.

No sex-related differences were found within samples. However, differences in the blood leukocyte profile between males and females were observed across samples. Leukocyte parameters of males from the studied samples differed in most indices, except for the proportions of lymphocytes, band neutrophils, and segmented neutrophils. In females, shifts in the leukocyte formula were manifested only in the monocyte lineage. Specifically, the proportion of monocytes in the blood of female marsh frogs from the extremely polluted water body was 2.5 times higher than that in females ($D$ = = 3.82, $p$ = 0.0007) inhabiting the water body of quality class 4 (Table 5).

*Integral leukocyte indices*

No sex-related differences were found in integral parameters. Furthermore, it should be noted that most integral leukocyte indices (blood cell index, *BCI*; neutrophil reactive response, *RNR*; lymphocyte-granulocyte index, *LGI*; neutrophil-lymphocyte ratio, *INLR* and neutrophil-eosinophil ratio, *INER*) also did not differ between the samples (Table 6), illustrating a similar level of systemic inflammation and immune response in frogs inhabiting conditions of environmental stress.

*Table 3*

**Prevalence of intracellular hemoparasites in *Pelophylax ridibundus* from Astrakhan Oblast**

| Samples | Number of infected individuals | Number of parasites per 500 erythrocytes | Proportion of parasitized cells | *E*, % | *I*, rel. units | *AI*, rel. units |
|---|---|---|---|---|---|---|
| Sample 1 | | | | | | |
| Females (*n* = 11) | 10 | 45 | 0.09 | 90.9 | 4.5 | 4.1 |
| Males (*n* = 11) | 10 | 69 | 0.138 | 90.9 | 6.9 | 6.3 |
| Total (*n* = 22) | 20 | 114 | 0.228 | 90.9 | 5.7 | 5.2 |
| Sample 2 | | | | | | |
| Females (*n* = 3) | 3 | 20 | 0.04 | 100.0 | 6.7 | 6.7 |
| Males (*n* = 19) | 16 | 58 | 0.116 | 84.2 | 3.6 | 3.1 |
| Total (*n* = 22) | 19 | 78 | 0.156 | 86.4 | 4.1 | 3.5 |

*Note: E* – extensiveness of invasion, *I* – average invasion intense, *AI* – abundance index, *n* – sample size, individuals

*Table 4*

**Functional activity of immune responses in infected *Pelophylax ridibundus***

| Indicator | Individuals | Sample, *Me/IQR* | | Statistical index |
|---|---|---|---|---|
| | | 1 | 2 | |
| Large immune complexes, optical density units/10 ml | Females (♀) | 34.02/28.5 | 26.58/20.04 | ***H* = 11.33, *p* = 0.01;**<br>$D_{1♀-2♀}$ = 2.06, *p* = 0.23;<br>$D_{1♀-1♂}$ = 0.39, *p* = 1.00;<br>$D_{1♀-2♂}$ = 1.50, *p* = 0.79;<br>$D_{1♂-2♂}$ = 1.93, *p* = 0.31;<br>$D_{1♂-2♀}$ = 1.79, *p* = 0.43;<br>**$D_{2♂-2♀}$ = 3.12, *p* = 0.01** |
| | Males (♂) | 29.8/14.5 | 37.47/10.68 | |
| Small immune complexes, optical density units/10 ml | Females (♀) | 69.3/46.2 | 44.6/6.24 | ***H* = 19.54, *p* = 0.0002;**<br>$D_{1♀-2♀}$ = 2.16, *p* = 0.18;<br>$D_{1♀-1♂}$ = 1.61, *p* = 0.63;<br>$D_{1♀-2♂}$ = 2.04, *p* = 0.24;<br>**$D_{1♂-2♂}$ = 3.83, *p* = 0.0007;**<br>**$D_{1♂-2♀}$ = 3.27, *p* = 0.006;**<br>$D_{2♂-2♀}$ = 0.95, *p* = 1.00 |
| | Males (♂) | 86.9/36.3 | 51.51/36.42 | |
| The ratio of large immune complexes to small immune complexes, rel. un. | Females (♀) | 0.52/0.35 | 0.64/0.49 | ***H* = 33.47, *p* < 0.001;**<br>$D_{1♀-2♀}$ = 0.12, *p* = 1.00;<br>$D_{1♀-1♂}$ = 1.70, *p* = 0.53;<br>**$D_{1♀-2♂}$ = 3.59, *p* = 0.001;**<br>**$D_{1♂-2♂}$ = 5.49, *p* < 0.001**;<br>$D_{1♂-2♀}$ = 1.02, *p* = 1.00;<br>$D_{2♂-2♀}$ = 2.44, *p* = 0.08 |
| | Males (♂) | 0.34/0.06 | 0.79/0.38 | |

*Note: Me/IQR* – median/interquartile range*; U* – the Mann – Whitney criterion; *H* – the Kruskal – Wallis criterion; *D* – the Dunn criterion; *p* – significance level. Significant differences are highlighted in bold.

*Table 5*

**Leukocyte profile of *Pelophylax ridibundus* infected with hemoparasites**

| Indicator | Individuals | Sample, *Me*/*IQR* | | Statistical index |
|---|---|---|---|---|
| | | 1 | 2 | |
| Myelocytes | Females (♀) | 6.0/6.0 | 3.0/3.0 | ***H* = 18.17, *p* = 0.0004;** |
| | Males (♂) | 4.0/2.5 | 6.5/6.0 | $D_{1♀-2♀}$ = 1.19, *p* = 1.00;<br>$D_{1♀-1♂}$ = 1.84, *p* = 0.38;<br>$D_{1♀-2♂}$ = 1.89, *p* = 0.34;<br>**$D_{1♂-2♂}$ = 3.94, *p* = 0.0004**;<br>$D_{1♂-2♀}$ = 0.06, *p* = 1.00;<br>$D_{2♂-2♀}$ = 2.46, *p* = 0.08 |
| Juvenile neutrophils | Females (♀) | 9.0/5.0 | 12.5/5.0 | ***H* = 41.88, *p* < 0.001;** |
| | Males (♂) | 6.0/4.0 | 15.5/9.5 | $D_{1♀-2♀}$ = 1.67, *p* = 0.56;<br>$D_{1♀-1♂}$ = 0.94, *p* = 1.00;<br>**$D_{1♀-2♂}$ = 4.76, *p* =0.00001;**<br>**$D_{1♂-2♂}$ = 5.81, *p* <0.001;**<br>$D_{1♂-2♀}$ = 2.31, *p* = 0.12;<br>$D_{2♂-2♀}$ = 1.30, *p* = 1.00 |
| Band neutrophils | Females (♀) | 2.0/3.0 | 2.5/4.0 | *H* = 0.99, *p* =0.80 |
| | Males (♂) | 2.0/2.0 | 2.0/2.5 | |
| Segmented neutrophils | Females (♀) | 3.0/2.0 | 3.0/3.0 | *H* = 1.48, *p* =0.68 |
| | Males (♂) | 3.0/3.0 | 3.0/1.5 | |
| Eosinophils | Females (♀) | 3.0/3.0 | 2.5/5.0 | ***H* = 18.98, *p* = 0.0003;** |
| | Males (♂) | 3.0/4.0 | 2.0/1.5 | $D_{1♀-2♀}$ = 0.98, *p* = 1.00;<br>$D_{1♀-1♂}$ = 0.33, *p* = 1.00;<br>**$D_{1♀-2♂}$ = 3.36, *p* = 0.004;**<br>**$D_{1♂-2♂}$ = 3.73, *p* = 0.001;**<br>$D_{1♂-2♀}$ = 1.21, *p* = 1.00;<br>$D_{2♂-2♀}$ = 1.12, *p* = 1.00 |
| Basophils | Females (♀) | 7.0/4.0 | 8.5/5.0 | ***H* = 15.06, *p* = 0.001;** |
| | Males (♂) | 7.0/2.5 | 4.0/2.5 | $D_{1♀-2♀}$ = 0.50, *p* = 1.00;<br>$D_{1♀-1♂}$ = 0.58, *p* = 1.00;<br>**$D_{1♀-2♂}$ = 2.67, *p* = 0.04;**<br>**$D_{1♂-2♂}$ = 3.32, *p* = 0.005;**<br>$D_{1♂-2♀}$ = 0.10, *p* = 1.00;<br>$D_{2♂-2♀}$ = 2.24, *p* = 0.15 |
| Monocytes | Females (♀) | 10.0/4.0 | 4.0/4.0 | ***H* = 35.35, *p* < 0.001;** |
| | Males (♂) | 8.5/5.0 | 5.5/2.5 | **$D_{1♀-2♀}$ = 3.82, *p* = 0.0007;**<br>$D_{1♀-1♂}$ = 1.91, *p* = 0.33;<br>**$D_{1♀-2♂}$ = 5.34, *p* =0.00001;**<br>**$D_{1♂-2♂}$ = 3.21, *p* = 0.007;**<br>$D_{1♂-2♀}$ = 2.52, *p* = 0.06;<br>$D_{2♂-2♀}$ = 0.58, *p* = 1.00 |
| Lymphocytes | Females (♀) | 60.0/11.0 | 65.5/10.0 | *H* = 7.40, *p* =0.06 |
| | Males (♂) | 63.5/4.5 | 59.5/14.0 | |

*Table 6*

**Integral leukocyte indices of *Pelophylax ridibundus* infected with hemoparasites**

| Indicator | Individuals | Sample, *Me/IQR* | | Statistical index |
|---|---|---|---|---|
| | | 1 | 2 | |
| Blood cell indicator (*BCI*), *rel. un.* | Females (♀) | 0.13/0.05 | 0.14/0.04 | $H$ = 2.35, $p$ =0.50 |
| | Males (♂) | 0.13/0.06 | 0.11/0.06 | |
| *R*eactive neutrophil response (*RNR*), *rel. un.* | Females (♀) | 0.02/0.04 | 0.04/0.07 | $H$ = 2.89, $p$ =0.40 |
| | Males (♂) | 0.02/0.04 | 0.04/0.17 | |
| Lymphocyte-Granulocyte index (*LGI*), *rel. un.* | Females (♀) | 63.0/31.26 | 65.41/21.59 | $H$ = 3.44, $p$ =0.32 |
| | Males (♂) | 64.72/29.42 | 79.19/49.58 | |
| Index of the neutrophil–lymphocyte ratio (*INLR*), *rel. un* | Females (♀) | 0.08/0.07 | 0.09/0.03 | $H$ = 0.22, $p$ =0.97 |
| | Males (♂) | 0.10/0.06 | 0.08/0.07 | |
| Index of the lymphocyte–eosinophil ratio (*ILER*), *rel. un* | Females (♀) | 19.67/10.37 | 26.0/42.8 | ***H* = 12.14, *p* = 0.007;**<br>$D_{1♀\text{-}2♀}$ = 1.25, $p$ = 1.00;<br>$D_{1♀\text{-}1♂}$ = 0.01, $p$ = 1.00;<br>**$D_{1♀\text{-}2♂}$ = 2.87, *p* = 0.02;**<br>**$D_{1♂\text{-}2♂}$ = 2.85, *p* = 0.02;**<br>$D_{1♂\text{-}2♀}$ = 1.24, $p$ = 1.00;<br>$D_{2♂\text{-}2♀}$ = 0.52, $p$ = 1.00 |
| | Males (♂) | 19.33/16.73 | 31.75/33.62 | |
| Index of the neutrophil–eosinophil ratio (*INER*), *rel. un* | Females (♀) | 1.33/1.41 | 2.5/3.83 | $H$ = 7.00, $p$ =0.07 |
| | Males (♂) | 1.40/1.95 | 3.0/4.66 | |
| *Leukocyte Shift* Index leukocyte shift index (*LSI*), *rel. un.* | Females (♀) | 0.34/0.15 | 0.42/0.16 | ***H* = 11.97, *p* = 0.0075;**<br>$D_{1♀\text{-}2♀}$ = 1.24, $p$ = 1.00;<br>$D_{1♀\text{-}1♂}$ = 0.77, $p$ = 1.00;<br>$D_{1♀\text{-}2♂}$ = 2.28, $p$ =0.13;<br>**$D_{1♂\text{-}2♂}$ = 3.14, *p* = 0.009;**<br>$D_{1♂\text{-}2♀}$ = 1.77, $p$ = 0.45;<br>$D_{2♂\text{-}2♀}$ = 0.15, $p$ = 1.00 |
| | Males (♂) | 0.33/0.13 | 0.45/0.28 | |
| Neutrophil nuclear shift index (*NNSI*), *rel. un.* | Females (♀) | 4.25/3.21 | 3.75/1.0 | ***H* = 26.50, *p* < 0.001;**<br>$D_{1♀\text{-}2♀}$ = 0.30, $p$ = 1.00;<br>$D_{1♀\text{-}1♂}$ = 1.09, $p$ = 1.00;<br>**$D_{1♀\text{-}2♂}$ = 3.59, *p* =0.001;**<br>**$D_{1♂\text{-}2♂}$ = 4.79, *p* =0.00001;**<br>$D_{1♂\text{-}2♀}$ = 1.00, $p$ = 1.00;<br>$D_{2♂\text{-}2♀}$ = 1.82, $p$ = 0.40 |
| | Males (♂) | 3.55/2.75 | 8.83/9.66 | |
| Leukocyte Intoxication Index (*LII*), *rel. un.* | Females (♀) | 0.27/0.22 | 0.26/0.04 | ***H* = 23.44, *p* < 0.001;**<br>$D_{1♀\text{-}2♀}$ = 0.58, $p$ = 1.00;<br>$D_{1♀\text{-}1♂}$ = 1.30, $p$ = 1.00;<br>**$D_{1♀\text{-}2♂}$ = 3.15, *p* = 0.009;**<br>**$D_{1♂\text{-}2♂}$ = 4.59, *p* = 0.00002;**<br>$D_{1♂\text{-}2♀}$ = 1.47, $p$ = 0.84;<br>$D_{2♂\text{-}2♀}$ = 1.40, $p$ = 0.95 |
| | Males (♂) | 0.19/0.10 | 0.39/0.36 | |

The increased relative number of eosinophils in the blood of individuals from the first sample was reflected in lower lymphocyte-eosinophil ratio (*ILER*) values compared to males from the second sample. The leukocyte intoxication index (*LII*), reflecting the overall body response, was higher in males of the second sample compared to males ($D = 4.59$, $p = 0.000021$) and females ($D = 3.15$, $p = 0.009$) of the first sample.

**Discussion**

This study evaluates adaptive responses of the blood system in marsh frogs infected with hemoparasites and inhabiting conditions of chemical pollution in two water bodies of the Lower Volga region. It is known that under natural conditions, selection tends to maintain an equal sex ratio in populations to maximize reproductive success. However, in anthropogenically transformed landscapes, a shift in abundance toward females is often recorded (Spirina, 2007; Lambert *et al.*, 2015). In our study, the first sample showed a strictly equal sex ratio despite extreme water pollution (water quality class 5), indicating the preservation of population homeostasis mechanisms. In the second sample (water quality class 4), a sex bias toward males was observed, apparently due to a number of factors, including reproductive behavior, social aspects, ecological interactions, and environmental conditions.

Microscopic identification of parasites in blood smears of marsh frogs revealed infection with hemoparasites of the genera *Dactylosoma* and *Hepatozoon*, as well as extracellular parasites (class Kinetoplastea, family Trypanosomatidae, *Trypanosoma* sp.; class Nematoda, family Onchocercidae, microfilariae larvae). The dominant hemoparasite was *Dactylosoma* sp., while microfilariae larvae were extremely rare. Representatives of the genera *Hepatozoon*, *Trypanosoma*, and *Lankesterella* showed moderate prevalence ranging from 18% to 45%. No significant sex-related differences in hemoparasite prevalence or parasitemia were detected, consistent with data from other researchers (Saglam & Arikan, 2006; Pavl'akova *et al.*, 2024).

It is known that blood parasites of the family Dactylosomatidae actively destroy mature erythrocytes in frogs during reproduction (merogony), leading to chronic anemia, to which the host organism responds with enhanced erythropoiesis, resulting in the release of immature polychromatophilic erythrocytes into the bloodstream and cell division within the circulatory system (Muñoz *et al.*, 2021). Infection modulates the immune system, maintaining it in a constant state of inflammation (Netherlands *et al.*, 2020a), which is manifested by alterations in the leukocyte profile. The increase in total leukocyte count and eosinophil proportion in infected individuals confirmed the classical organismal response to parasitic aggression (Pavl'akova *et al.*, 2024; Romanova *et al.*, 2018; Romanova *et al.*, 2025). Marsh frogs inhabiting extreme conditions exhibited higher prevalence and intensity of infection, as well as a significant proportion of infected erythrocytes, indicating reduced organismal resistance and increased susceptibility to parasitic agents (Blaustein *et al.*, 2012; Koprivnikar & Marcogliese, 2011). The decrease in lymphocyte proportion coupled with an increase in integral indices (*LSI*, *BCI*) illustrates the transition of the organism into a state of physiological stress and possible depletion of the specific immune component (Zaripova & Faizulin, 2017; Gromov *et al.*, 2023).

Humoral immunity in amphibians, as in other vertebrates, is mediated by immunoglobulins (antibodies) – key effectors of the specific immune response. The elevated mean aggregation index (ratio of large to small immune complexes) in males inhabiting the less polluted aquatic environment (water quality class 4) indicated normal or active functioning of the blood immune system and significantly exceeded the values of males and females from the extremely polluted environment, suggesting successful elimination of antigen-antibody complexes from the organism. Infected marsh frogs inhabiting the extremely polluted aquatic environment (water quality class 5) were characterized by elevated levels of small circulating immune complexes, indicating both active immune inflammation and the possible development of pathological autoimmune and allergic reactions (Romanova & Nikolaev, 2011). Nota-

bly, the level of large immune complexes in the blood of marsh frogs from the extremely polluted environment (water quality class 5) was reduced. It can be assumed that under conditions of extreme pollution, the organism redirects its resources from antibody production to cellular defense mechanisms to more effectively counteract intoxication.

Quantitative and qualitative changes in blood leukocyte parameters were more pronounced in infected males compared to infected females, differing in total granulocyte and agranulocyte counts, neutrophil granulocyte counts, and most blood formula parameters, except for the proportions of lymphocytes, band neutrophils, and segmented neutrophils. Elevated monocyte counts against a background of decreased neutrophil proportion indicated intense immune system activity in males inhabiting extremely polluted waters. At the same time, the increased leukocyte shift index, *LSI* (males, sample 2) indicated a predominant role of granulocytes (due to young neutrophil granulocytes) at this stage of the immune response, which was confirmed by a high neutrophil nuclear shift index (*NNSI*) with a relative lag in the lymphocyte–monocyte cell lineage.

It is known that suppression of amphibian immune mechanisms under pollution conditions with enhanced inflammatory processes is observed during infections (Gervasi *et al.*, 2025) and accumulation of heavy metals (Zhelev *et al.*, 2020), high concentrations of which suppress phagocytosis and induce restructuring of the leukocyte profile (Jayawardena *et al.*, 2015). Adaptive immunity in amphibians is evolutionarily tuned to a slow response, characterized by low affinity and slow dynamics (Patel & Hsu, 2023); under conditions of aquatic ecosystem pollution, it becomes the primary target, forcing the organism to rely on innate defense mechanisms (Rollins-Smith, 2017; Robert, 2023). Lymphoid tissue cells are sensitive to toxic damage, and under unfavorable environmental conditions, antibody production is impaired (Robert, 2023), making this a less effective resource for the organism in extremely polluted environments compared to cellular responses. Thus, according to our results hemoparasitic invasion can acts as an additional factor modulating the immune response under environmental stress.

## Conclusion

The obtained results indicate a profound restructuring of the immune status of *P. ridibundus* under the influence of anthropogenic stress and infection with both intracellular (*Dactylosoma* sp., *Hepatozoon* sp., *Lankesterella* sp.) and extracellular (*Trypanosoma* sp., microfilariae larvae) hemoparasites. Males are more susceptible to infection than females. The leukocyte profile of infected individuals illustrates the transition of the organism into a phase of chronic stress with depletion of specific defense mechanisms and reduced organismal resistance. The predominance of small immune complexes against a background of large immune complex deficiency indicates an excess of antigens and the risk of developing autoimmune pathologies. Under conditions of environmental stress and hemoparasitic invasion, depletion of lymphoid resources occurs, and nonspecific cellular mechanisms become established as the dominant survival strategy in amphibians.

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## Declaration of Competing Interest

The authors declare no conflicts of interest.

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