

Comparative analysis of the cellular composition of hemolymph in pulmonate and prosobranch mollusks

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ABSTRACT: Despite numerous studies on the cellular composition of gastropod hemolymph, questions regarding cell classification and their functional heterogeneity remain debatable, especially in frame of comparative analysis of representatives from different systematic groups. This prompted us to conduct a comparative analysis of circulating cells in two species of freshwater snails — the pulmonate *Planorbarius corneus* and the prosobranch *Viviparus viviparus*. The study employed light microscopy, flow cytofluorimetry, and imaging cytometry. The presence of lysosomes, mitochondrial membrane potential, and spontaneous reactive oxygen species production in hemocytes were assessed using the fluorescent dyes LysoTracker (LT), MitoTracker (MT), and carboxy-H2DCFDA. In *P. corneus*, two hemocyte populations (A and B) were identified, while *V. viviparus* had three populations of hemocytes (A, AB, and B), which differed in size, granularity, presence of pseudopodia, and metabolic activity. An additional population C found in both mollusks was represented by cells at different stages of apoptosis. Analysis using LT showed that the presence of acidic granules correlates with high cell activity. In *P. corneus*, LT⁺ and LT[−] hemocyte populations were more discrete, indicating a clear separation of the active and the inactive forms. In *V. viviparus*, the ratio of such cells varies depending on the population, forming a gradient of functional activity. In *P. corneus*, hemocytes of population A were highly variable in respect of mitochondrial potential and production of reactive oxygen species, while hemocytes of population B were homogeneous by these characteristics. In *V. viviparus*, cells with different metabolic activity were present in all populations. The obtained data suggest that *P. corneus* is characterized by a linear pathway of hemocyte differentiation (from hyalinocytes to granulocytes). At the same time, the hemolymph of *V. viviparus* contains cells at different maturation stages, including transitional forms.

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Сравнительный анализ клеточного состава гемолимфы лёгочных и переднежаберных моллюсков

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РЕЗЮМЕ: Несмотря на многочисленные исследования клеточного состава гемолимфы гастропод, вопросы классификации и функциональной гетерогенности клеток остаются дискуссионными, особенно при сравнении представителей разных систематических групп. Это обусловило проведение нами сравнительного анализа циркулирующих клеток двух видов пресноводных улиток — пульмонат *Planorbarius corneus* и прозобранхий *Viviparus viviparus*. Для изучения использовали методы световой микроскопии, проточной цитофлуориметрии, а также цитометрии с визуализацией. Наличие лизосом, мембранный потенциал митохондрий и спонтанную продукцию активных форм кислорода в гемоцитах оценивали при помощи флуоресцентных красителей LysoTracker (LT), MitoTracker (MT) и карбокси-H₂DCFDA. У *P. corneus* выявлены две популяции гемоцитов (А и В), а у *V. viviparus* — три (А, АВ и В), которые различаются по размеру, гранулярности, наличию псевдоподий, а также по метаболической активности. У обоих моллюсков обнаружена дополнительная популяция С, которая представлена клетками, находящимися на разных стадиях апоптоза. Анализ с применением LT показал, что наличие кислых гранул коррелирует с высокой активностью клеток. У *P. corneus* популяции LT⁺ и LT⁻ гемоцитов более дискретны, что свидетельствует о чётком разделении на активные и неактивные формы. У *V. viviparus* соотношение таких клеток варьирует в зависимости от популяции, формируя градиент функциональной активности. В популяции А гемоцитов *P. corneus* выявлена значительная вариабельность уровней митохондриального потенциала и продукции активных форм кислорода. При этом в популяции В отмечена гомогенность клеток по этим характеристикам. У *V. viviparus* клетки с различной метаболической активностью присутствовали во всех популяциях. Полученные данные позволяют предположить, что у *P. corneus* реализуется линейный путь дифференцировки гемоцитов (от гиалиноцитов к гранулоцитам), в то время как гемолимфа *V. viviparus* содержит клетки на разных этапах созревания, включая переходные формы.

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КЛЮЧЕВЫЕ СЛОВА: гемолимфа, *Planorbarius corneus*, *Viviparus viviparus*, проточная цитофлуориметрия, проточная цитометрия с визуализацией.

Introduction

The study of the cellular composition of the molluscan hemolymph is important for understanding the mechanisms of innate immune response. Hemocytes are key effector cells that perform phagocytosis, produce cytotoxic substances such as reactive oxygen species (ROS) and nitrogen oxide, encapsulate foreign objects, etc. (Coustau *et al.*, 2015; Pila *et al.*, 2016; Tetreau *et al.*, 2017; Melillo *et al.*, 2018; Ataev *et al.*, 2020, 2024).

The importance of comparative analysis of the common freshwater mollusks *Planorbarius corneus* and *Viviparus viviparus* is mostly associated with their role as intermediate hosts of many trematode species. *Planorbarius corneus* is a model organism for the studies of the hemocyte responses to parasitic invasions. Two main types of hemocytes, granulocytes and hyalinocytes, previously identified in the hemolymph of these mollusks differ in granularity, nucleoplasmic ratio and adhesive ability (Ataev *et al.*, 2016; Prokhorova *et al.*, 2018a, b). It has been noted that infection results in a shift in the ratio of the cell populations towards granulocytes (Ataev *et al.*, 2016; Serebryakova *et al.*, 2022), which are involved in the formation of hemocyte agglutinations on the surface of sporocysts. Transcriptome analysis has confirmed that various immune response genes are activated in the hemocytes of *P. corneus* during natural infection with trematodes *Bilharziella polonica*, including pathogen recognition receptors, cytokines and other components of innate immunity (Orlov *et al.*, 2023).

Studies of the cellular composition of hemolymph of prosobranch mollusks, especially freshwater ones, are more scarce (Ottaviani, 1989; Ray *et al.*, 2013; Suwannatrai *et al.*, 2020; Li *et al.*, 2023; Serebriakova *et al.*, 2024). It has been shown that in *Viviparus ater* several populations of hemolymph cells are actively involved in the immune response, including the production of ROS (Ottaviani *et al.*, 1992). These observations agree with the data on other gastropod mollusks.

In this study we performed a morphometric and functional analysis of hemolymph cells of the pulmonate mollusk *Planorbarius corneus* and the prosobranch mollusk *Viviparus viviparus*. Methods were used which allowed a comprehensive

assessment of morphology and functional activity of circulating cells. Comparisons of species from different taxonomic groups will help us better understand the universality of gastropod immune response mechanisms.

Material and Methods

We used mollusks *Planorbarius corneus* Linnaeus, 1758 (Heterobranchia, Planorbidae) (n=100) and *Viviparus viviparus* Linnaeus, 1758 (Caenogastropoda, Viviparidae) (n=100). Snails *P. corneus* were collected in the Oredezh River near the village of Vyritsa (59°24'50"N, 30°20'50"E) in the Leningrad Region, Russia, in 2015. Snails *V. viviparus* were collected in the Gulf of Finland in the Lisy Nos area (60°00'54"N, 30°00'30"E) of the Kurortny District of St. Petersburg, Russia, in 2024.

The snails were kept in the laboratory in 5–10-liter water tanks with aerated water and sandy soil at room temperature (20–23°C). The light regime was 12:12. Males and females of *V. viviparus* were kept separately. The water was changed two or three times a week. The diet of *V. viviparus* consisted of pre-soaked dried willow leaves. Snails *P. corneus* were fed on fresh lettuce leaves.

The infection of mollusks with trematodes was preliminarily determined based on the emission of cercariae and confirmed by dissection of the snails after hemolymph collection. Hemolymph from uninfected mollusks was used for hemocyte analysis. Hemolymph was collected from the pericardium of the snails using a glass Pasteur pipette.

Morphological analysis

Microscopic analysis was performed on smears of freshly collected hemolymph on glass slides with an adhesive polylysine coating. The hemolymph was incubated for 30 min in a humid chamber at room temperature. The preparations were examined under a Leica DM 5000 microscope using phase contrast (PH) and differential interference contrast (DIC).

Cytofluorimetric analysis

Cytofluorimetric analysis was performed immediately after hemolymph collection. Hemocyte populations were isolated based on the values of forward scatter (FSC) and side scatter (SSC), which characterize the size and the complexity of the cell structure, respectively. The ratio of each population was expressed as a percentage of the total number of single hemocytes.

Lysosomotropic dye LysoTrackerTM Green DND-26 (Thermo Fisher Scientific, USA) was used to stain lysosomes. This dye penetrates the plasma membrane and accumulates in granules with pH < 6. The dye was added to hemolymph samples at a final concentration of 100 nM, incubated in the dark for 10 min and then analyzed by flow cytometry. The intensity of

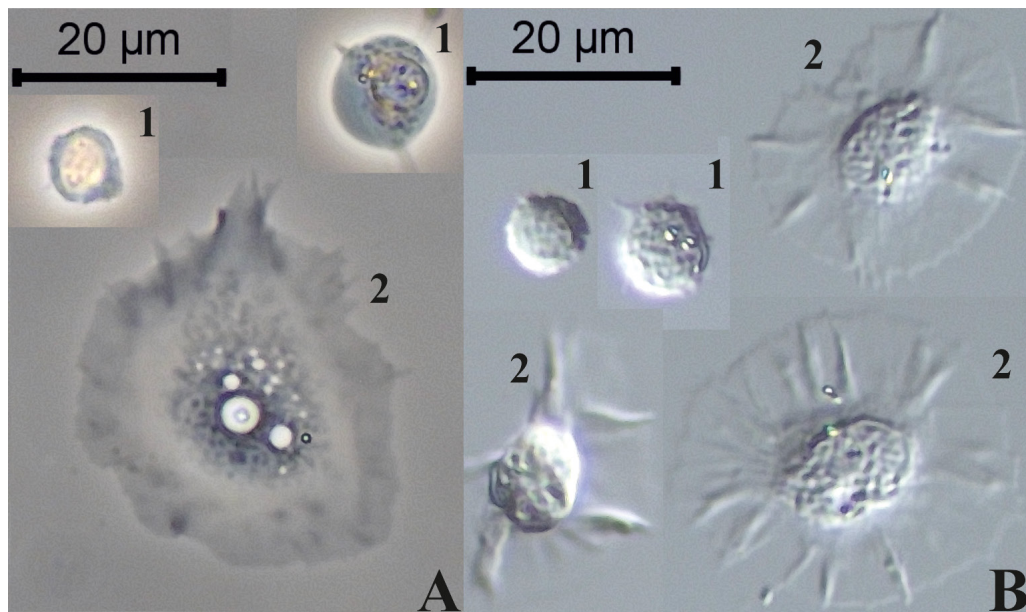


Fig. 1. Hemocytes of *Planorbarius corneus*. A — phase-contrast microscopy; B — differential interference contrast microscopy. 1 — hyalinocyte; 2 — granulocyte.

LysoTracker fluorescence was determined for each hemocyte population. Subpopulations with a high (LT^+) and low (LT^-) dye fluorescence intensity were identified within each population. For each subpopulation, FSC and SSC values of the hemocytes were also determined.

For specific staining of mitochondria, we used fluorescent dye MitoTracker™ Red CMXRos (Thermo Fisher Scientific, USA), which penetrates intact cells and binds to mitochondrial lipids and proteins (Buravkov *et al.*, 2014) in a potential-dependent manner (Pendergrass *et al.*, 2004). The dye was added to hemolymph samples at a final concentration of 200 nM, incubated in the dark for 10 min and then analyzed by flow cytometry. The intensity of MitoTracker fluorescence was determined for each hemocyte population. Subpopulations with a high (MT^+), medium (MT^-) and low (MT^-) dye fluorescence intensity were identified within each population. For each subpopulation, FSC and SSC values of the hemocytes were also determined.

To determine the level of spontaneous production of ROS by hemocytes, the dye 6-Carboxy-H2DCFDA (6-Carboxy-2',7'-dichlorodihydrofluorescein diacetate) (Lumiprob RUS, Russia) was used. This dye penetrates the intact plasma membrane and becomes fluorescent as a result of the oxidation of ROS in the cytoplasm (Diaz *et al.*, 2003). It was added to hemolymph samples at a final concentration of 10 μ M, after which the samples were incubated for 30 min in the

dark. A propidium iodide (PI) solution was then added at a final concentration of 5 μ g/ml in order to remove damaged and dead cells from the analysis zone. The analysis was performed using a flow cytometer. The fluorescence intensity of the ROS probe was assessed for PI-hemocytes of each population. Subpopulations with a high (ROS^+) and low (ROS^-) fluorescence intensity could be distinguished in the populations.

Sample analysis was performed using a BD Accuri™ C6 flow cytometer (BD Biosciences, USA). At least 50 μ l of hemolymph were analyzed in each sample. The samples were analyzed separately for each snail; hemolymph samples were not pooled. The results were processed using Kaluza software™ v.2.0 (Beckman Coulter, USA) and FlowJo™_v.10.10.0 (BD Bioscience, USA).

Imaging flow cytometry

Imaging flow cytometry was performed by a flow cytometer with the function of statistical quantitative analysis of cell images Amnis® ImageStream® System (Cytek® Bioscience, USA). This approach makes it possible to obtain cytometric data about a hemolymph sample and simultaneously to visualize events on a cytogram. Signals characterizing cell morphology and complexity (Ch01 and Ch06 channels) and fluorescence (LysoTracker by Ch02 channel) were registered. Not less than 1,000 events, excluding calibration beads, were analyzed for each sample. Data processing was performed using IDEAS 6.2 software (Cytek® Bioscience, USA). Before this analysis the

samples were stained with LysoTracker (see above). Hemocyte populations were isolated based on cell area (bright field) and SSC values (dark field). Since it was possible to compare the histograms describing the LysoTracker fluorescence in hemocytes with the images of cells distributed into different areas, we could identify different cell groups within the populations fairly accurately. In this way, we identified subpopulations accumulating LysoTracker (LT⁺) and subpopulations not accumulating this dye (LT⁻). The intensity of LysoTracker fluorescence, SSC value and cell diameter were evaluated for each subpopulation.

Statistical analysis

Statistical processing and graphical presentation of data were performed using MS Excel (Microsoft, USA) and GraphPad Prism 6.01 (GraphPad Software Inc., USA). The normality of distribution in the samples was assessed using the Kolmogorov-Smirnov criterion. The results were presented as the median (Me) and the interquartile range (Q25, Q75) (for proportions also as mean $\pm$ standard error, $\bar{X} \pm SE$). Multiple comparisons of populations were performed using the Kruskal-Wallis test followed by pairwise comparisons using Dunn's test.

Results

Planorbarius corneus

Microscopic examination showed that there were two types of cells in the hemolymph of *P. corneus*: granulocytes and hyalinocytes (Fig. 1). Granulocytes were larger and easily spread on the substrate, forming numerous filopodia. Hyalinocytes were rounded and could form lobopodia.

Two populations of hemocytes were also identified in the hemolymph of *P. corneus* based on cytometric analysis (Fig. 2): A — relatively small cells with a simple structure of the surface and the cytoplasmic apparatus; B — larger cells with a more complex structure. Medium-sized cells with high SSC values (population C) were also seen in the cytograms. We have previously established that this area contains cells at different stages of apoptosis (Tokmakova *et al.*, 2020; Serebryakova *et al.*, 2022).

Hemocytes of population A predominated in the hemolymph of *P. corneus*, making up, on average, 65%. Hemocytes of population B made up about 24% (Table 1). The remaining 11% of hemocytes belong to population C.

The highest level of LysoTracker accumulation was observed in the hemocytes of population B. The fluorescence level in this group was

$1.58 \cdot 10^5$ ($1.33 \cdot 10^5$; $2.06 \cdot 10^5$), which is 1.5 times higher than the corresponding values in population A ($1.01 \cdot 10^5$ [$0.84 \cdot 10^5$; $1.32 \cdot 10^5$], $p < 0.005$) and population C ($1.13 \cdot 10^5$ [$0.94 \cdot 10^5$; $1.63 \cdot 10^5$], $p < 0.005$). At the same time, populations A and B were divided into two subpopulations, LT⁺ and LT⁻, which differed significantly in LysoTracker fluorescence levels ($p < 0.005$).

In population A, 93.7% (77.45; 97.55) of the cells had a low level of LysoTracker fluorescence ($0.96 \cdot 10^5$ [$0.82 \cdot 10^5$; $1.18 \cdot 10^5$]), while 6.82% (2.76; 21.95) of the cells had a high level of LysoTracker fluorescence ($1.85 \cdot 10^5$ [$1.52 \cdot 10^5$; $2.31 \cdot 10^5$]). Conversely, in population B, LT⁻ subpopulation with the fluorescence level of $0.79 \cdot 10^5$ ($0.67 \cdot 10^5$; $1.38 \cdot 10^5$) made up 4.1% (1.69; 17.15), while 95.6% (82.55; 98.25) of the cells belonged to LT⁺ subpopulation with the fluorescence level of $1.97 \cdot 10^5$ ($1.38 \cdot 10^5$; $2.36 \cdot 10^5$). At the same time, the hemocytes of these two subpopulations did not differ significantly from each other in terms of FSC and SSC.

MitoTracker staining showed that in *P. corneus* the hemocytes of the two populations accumulated this dye with different efficiency. The maximum fluorescence was observed in population B and made up $7.82 \cdot 10^4$ ($5.29 \cdot 10^4$; $10.57 \cdot 10^4$). In population A, the fluorescence was significantly lower: $2.93 \cdot 10^4$ ($2.33 \cdot 10^4$; $4.60 \cdot 10^4$) ($p < 0.01$). In addition, three separate subpopulations could be distinguished within each population based on the level of MitoTracker fluorescence, though they did not differ in terms of FSC and SSC (Fig. 3).

In population A, hemocytes with the maximum level of MitoTracker fluorescence made up 62.15% (44.48; 83.43), those with the medium level made up 15.60% (10.14; 31.65), and those with a weak fluorescence made up 10.28% (4.60; 25.45). In population B, the percentage of hemocytes with a high level of MitoTracker fluorescence was greater: 86.80% (80.50; 93.75). Also, 9.91% (4.59; 17.35) of hemocytes in population B showed a medium fluorescence and 2.36% (1.22; 5.52) showed a weak fluorescence.

When hemocytes of *P. corneus* were stained with a fluorescent ROS probe, two subpopulations were identified in population A, differing in the level of spontaneous ROS production. Low levels of ROS production were characteristic of 81.57% (67.54; 94.20) of the cells, while in 14.75% (5.52; 27.46) of the cells the levels of

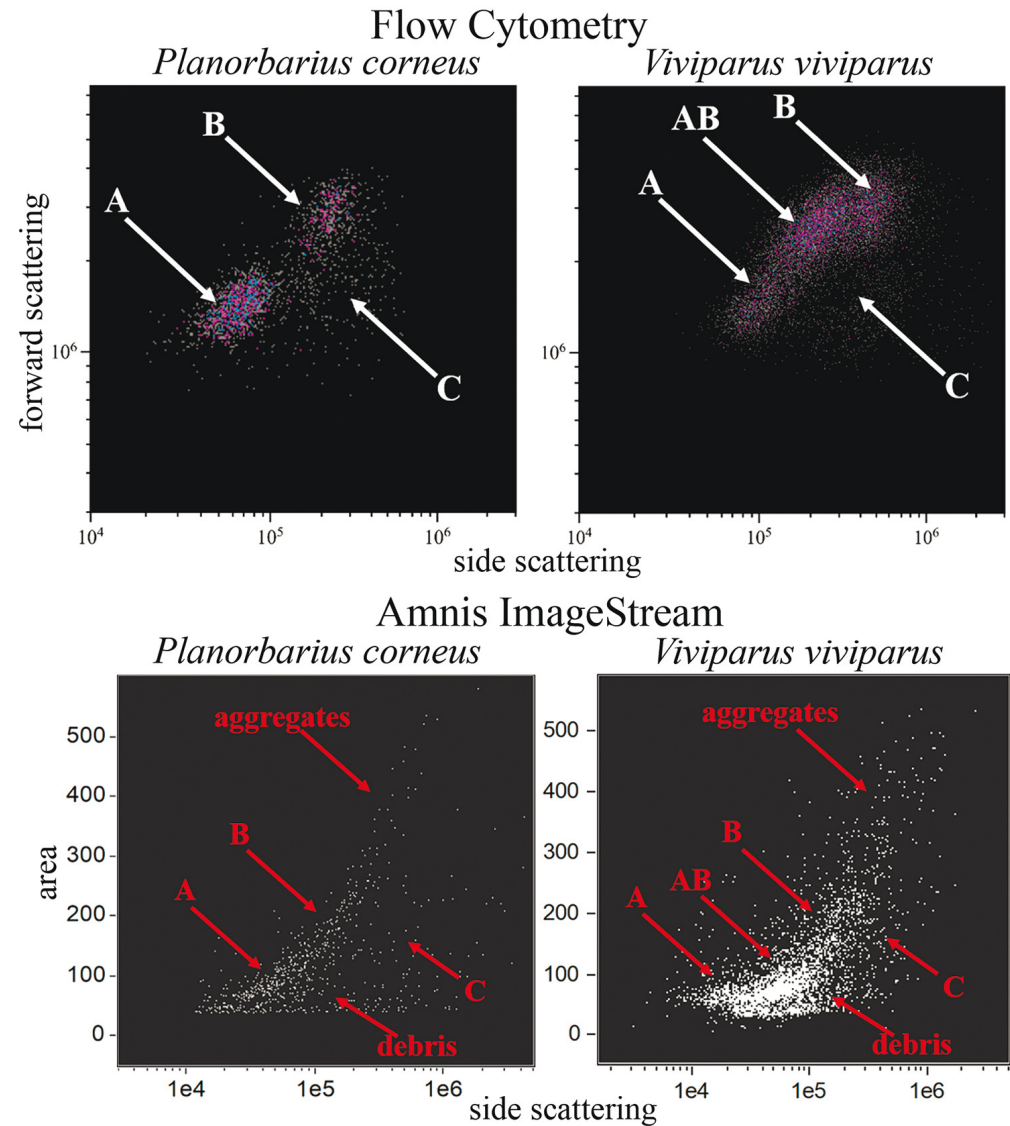


Fig. 2. Algorithm for the identification of circulating hemocyte populations in *Planorbarius corneus* and *Viviparus viviparus* based on the parameters characterizing cell size (area, FSC) and structural complexity (SSC).

ROS production were an order of magnitude higher. At the same time, hemocytes with high levels of ROS production did not differ significantly in size and complexity of the cytoplasmic compartment from the other cells in population A. Hemocytes in population B produced twice as much ROS as those in population A.

Cytometric analysis with visualization (ImageStream) showed the presence of five areas on

the histograms for *P. corneus* (Fig. 2). The smallest objects, approximately 2–3 μm in diameter, were debris and calibration beads. A clearly distinguished group of small objects was population A. This area of the histogram included rounded cells with a diameter of 11.13 (10.52; 12.03) μm , which had short pseudopodia or no pseudopodia at all.

Larger objects with a diameter of 15.24 (14.23; 16.38) μm were considered as cells

Table 1. Frequency of hemocytes' different populations in the hemolymph of *Planorbarius corneus* and *Viviparus viviparus*.

	A	AB	B	C
<i>Planorbarius corneus</i> , n=27	68.92 (54.46;82.24) / 64.82 ± 3.97	–	22.17 (12.15;31.73) / 23.99 ± 3.02	7.93 (3.74;14.79) / 11.06 ± 2.14
<i>Viviparus viviparus</i> , n=44	16.33 (11.41;19.45) / 15.95 ± 0.95	19.32 (15.77;22.48) / 19.28 ± 0.80	55.16 (50.60;63.70) / 56.24 ± 1.64	7.60 (6.29;9.52) / 8.54 ± 0.47

of population B. These hemocytes had a more complex structure and formed pseudopodia. However, large rounded cells with granules and no pseudopodia were also noted in this area of the histograms. The upper right area contained the largest and the most complex objects, namely, cell aggregates, while the area designated as population C contained cells at various stages of apoptosis, cell fragments and large debris.

LysoTracker staining proved that cells containing acidic lysosomes were present in both cell populations (Fig. 4). In population A, 72.64%±6.38 of the hemocytes were not stained by LysoTracker, while in population B lysosome-containing cells predominated (67.15%±12.14). Hemocytes that did not stain with LysoTracker could be morphologically different but were usually represented by rounded cells without pseudopodia. Hemocytes containing lysosomes always had pseudopodia but their size and number varied.

Comparison of LT⁻ and LT⁺ hemocytes did not reveal any statistically significant differences in the area of cells between populations A and B. In population A, LT⁺ hemocytes had a much higher SSC than LT⁻ hemocytes. However, SSC of hemocytes from population A was lower than that of hemocytes from population B.

Viviparus viviparus

The hemolymph cells of *V. viviparus* differed in size, ability to form pseudopodia and their shape, the presence of granules and vacuoles in the cytoplasm and other characteristics. Based on the microscopic analysis, they were divided into three main types (Fig. 5). Cells of type I were small and lacked pseudopodia, granules and vacuoles. Cells of type II were particularly variable morphologically. They could form pseudopodia of various shape, and small granules and vesicles were visible in their cytoplasm.

Cells of type III were the largest hemocytes. They could spread on the substrate, forming numerous pseudopodia.

Three populations of hemocytes (A, AB, and B) were usually observed in cytograms of *V. viviparus* hemolymph based on FSC and SSC (Fig. 2). The remaining cells were designated, as in case of *P. corneus*, as population C. Hemocytes of population B predominated in the hemolymph (55%), while populations A and AB were represented by an approximately equal percentage (16% and 19%, respectively) of the total number of hemocytes. The remaining 8.5% of the hemocytes were placed in area C.

The maximum LysoTracker accumulation level was observed in hemocytes of population B, making up 4.05*10⁵ (2.81*10⁵; 4.60*10⁵), which was almost twice higher than the corresponding value in population AB, where it was 2.49*10⁵ (82*10⁵; 3.21*10⁵) (p<0.01). Hemocytes of population A accumulated LysoTracker weakly: 0.96*10⁵ (0.76*10⁵; 1.20*10⁵).

Each population contained some hemocytes with a high level of LysoTracker fluorescence and some hemocytes with a low level. In population A, 71.50% (59.88; 82.00) of hemocytes had low fluorescence levels (0.79*10⁵ [0.71*10⁵; 0.88*10⁵]), while 28.50% of hemocytes (18.00; 40.13) had a high fluorescence level (2.42*10⁵ [2.12*10⁵; 2.77*10⁵]). In population AB, 37.85% (26.48; 46.58) of hemocytes had low LysoTracker fluorescence levels (1.56*10⁵ [1.42*10⁵; 1.71*10⁵]), while 61.65% of hemocytes (53.23; 73.03) had a high level (3.80*10⁵ [3.55*10⁵; 4.21*10⁵]). In population B, 85.65% (78.60; 89.38) of hemocytes had a high level of LysoTracker fluorescence (4.68*10⁵ [4.25*10⁵; 5.27*10⁵]), while 13.80% (10.25; 20.88) of hemocytes had low fluorescence levels (1.56*10⁵ [1.44*10⁵; 1.81*10⁵]). These subpopulations differed in size only for cells of population B: FSC values for LT⁻ cells were

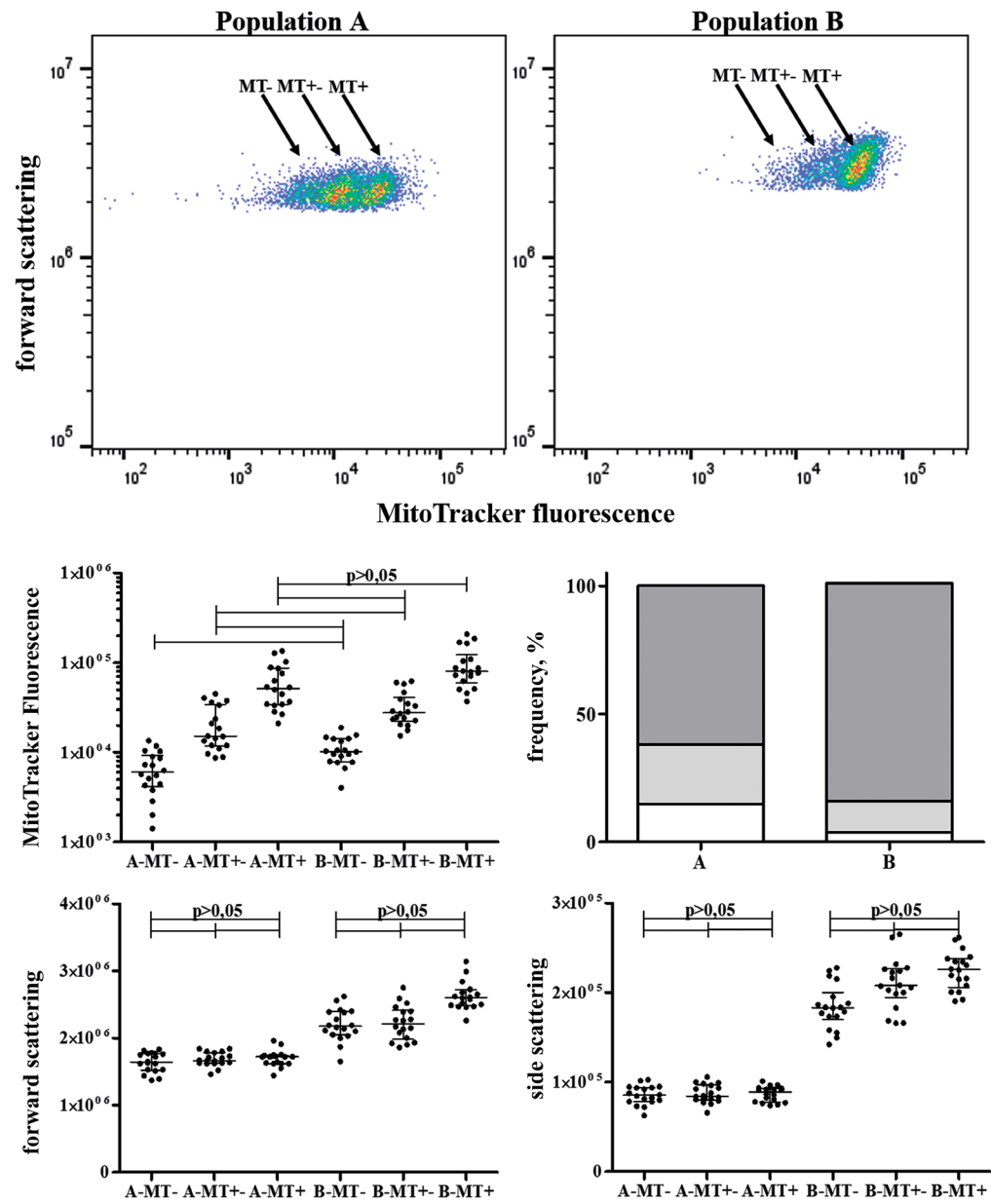


Fig. 3. MitoTracker fluorescence in different populations of hemocytes of *Planorbarius corneus*.

2.68×10^6 (2.54×10^6 ; 2.80×10^6), while the values for LT^+ were 3.00×10^6 (2.92×10^6 ; 3.09×10^6), ($p < 0.05$). No differences in SSC values between LT^+ and LT^- subpopulations were observed.

The intensity of MitoTracker fluorescence was different in all populations of *V. viviparus*

hemocytes. It was the greatest in population B, making up 1.55×10^4 (1.19×10^4 ; 2.41×10^4), and much lower in populations A and AB: 0.60×10^4 (0.41×10^4 ; 0.76×10^4) and 0.86×10^4 (0.66×10^4 ; 0.94×10^4), respectively ($p < 0.01$). There was no statistically significant difference between the

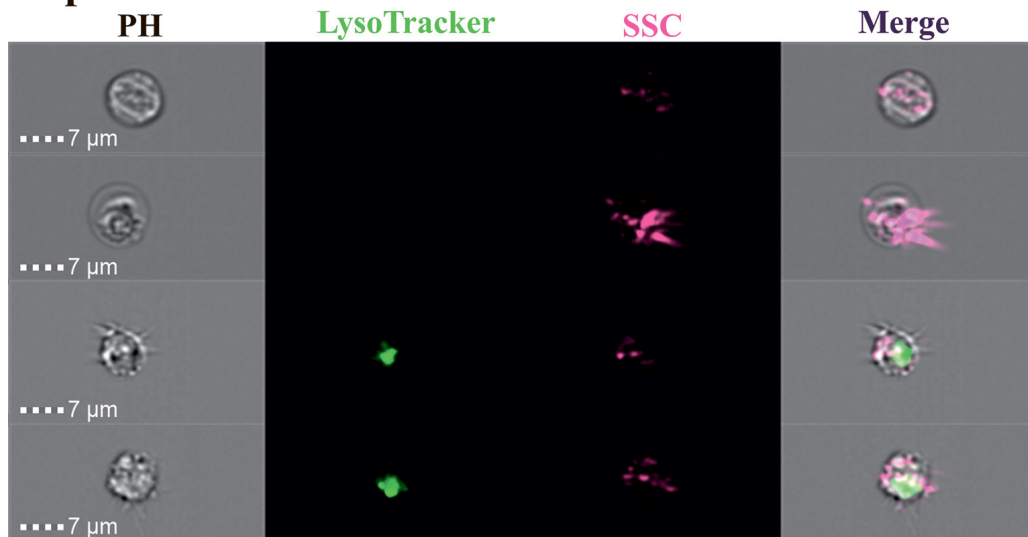
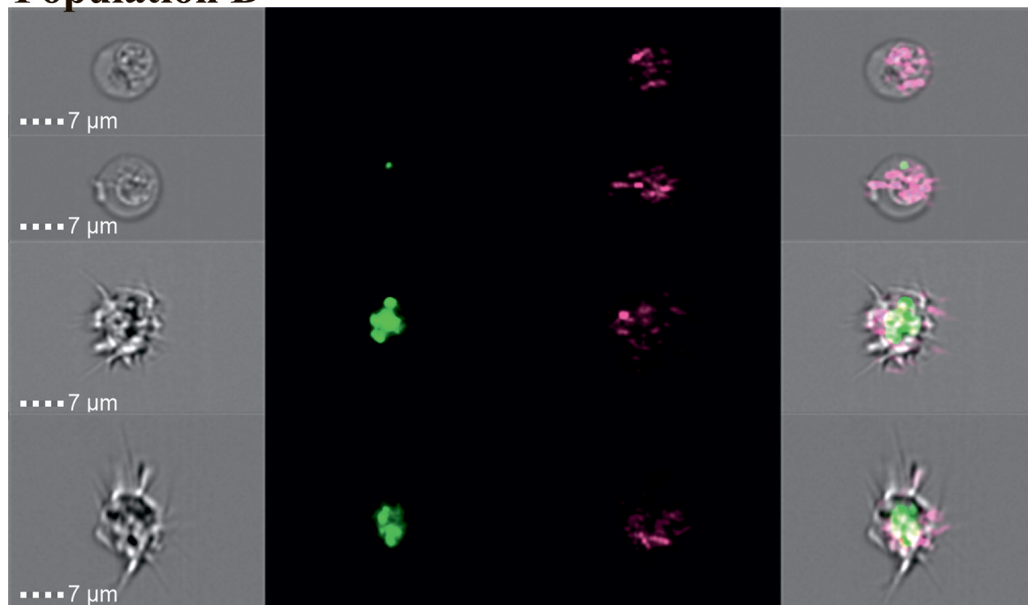
Population A**Population B**

Fig. 4. The analysis of circulating hemocytes in the snail *Planorbarius corneus* performed with the help of Amnis® ImageStream imaging flow cytometer. Accumulation of LysoTracker (green) and SSC (magenta) in hemocytes is shown. PH — phase contrast image of signals characterizing cell morphology.

intensity of MitoTracker fluorescence in population A and population AB.

Three separate subpopulations, not differing in terms of light scattering parameters, could be distinguished in each population based on MitoTracker fluorescence (Fig. 6).

Subpopulations with high, medium, and low MitoTracker fluorescence levels were represented approximately equally in all populations, the percentage being, on average, 50-55% for MT⁺⁺ hemocytes, 25-30% for MT⁺ hemocytes and 15% for MT⁻ hemocytes.

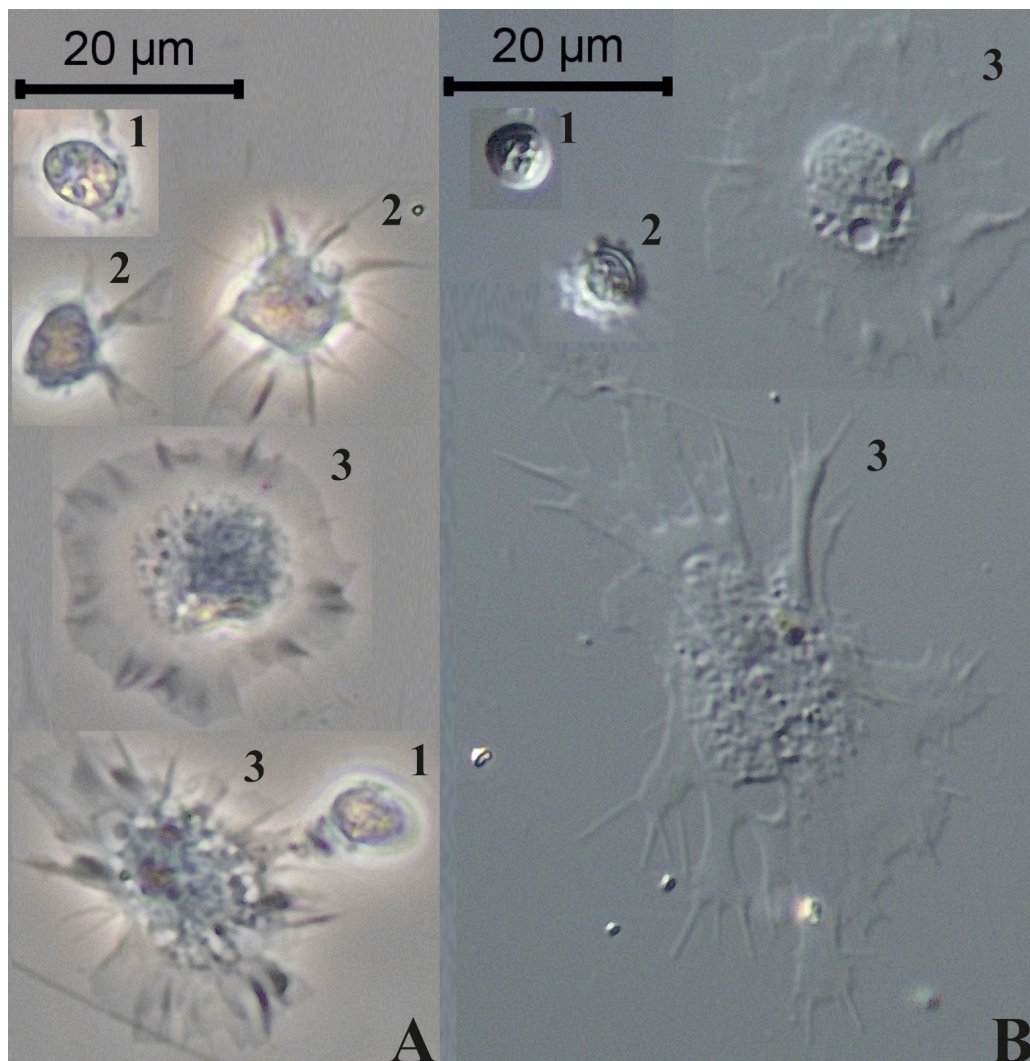


Fig. 5. Hemocytes of snails *Viviparus viviparus*. A — phase-contrast microscopy; B — differential interference contrast microscopy. 1 — hemocytes of type I; 2 — hemocytes of type II; 3 — hemocytes of type III.

The median of fluorescence, which characterizes the amount of ROS in the hemocytes of *V. viviparus*, was the lowest in population A ($3.10 \cdot 10^5$ [$1.82 \cdot 10^5$; $4.64 \cdot 10^5$]) and increased with the increasing size and complexity of the cells. In population AB, the median of fluorescence made up $5.41 \cdot 10^5$ ($2.66 \cdot 10^5$; $7.29 \cdot 10^5$), while in population B it made up $7.29 \cdot 10^5$ ($3.31 \cdot 10^5$; $9.44 \cdot 10^5$). At the same time, two separate subpopulations could be distinguished in each population, differing by more than an order of magnitude in the level of ROS production. The number of hemocytes with a high ROS

production made up 15.85% (11.61; 25.04) in population A, 12.93% (4.76; 17.07) in population AB and 3.20% (2.75; 10.79) in population B. Hemocytes with high levels of ROS production did not differ significantly from the other cells in their populations in terms of size and complexity of the cytoplasmic compartment.

The study of hemolymph using flow cytometry with visualization revealed six areas on cytograms (Fig. 2): small debris, hemocyte populations A, AB, and B, cell aggregates and area C, consisting of apoptotic cells and large debris. The diameter of hemocytes in popula-

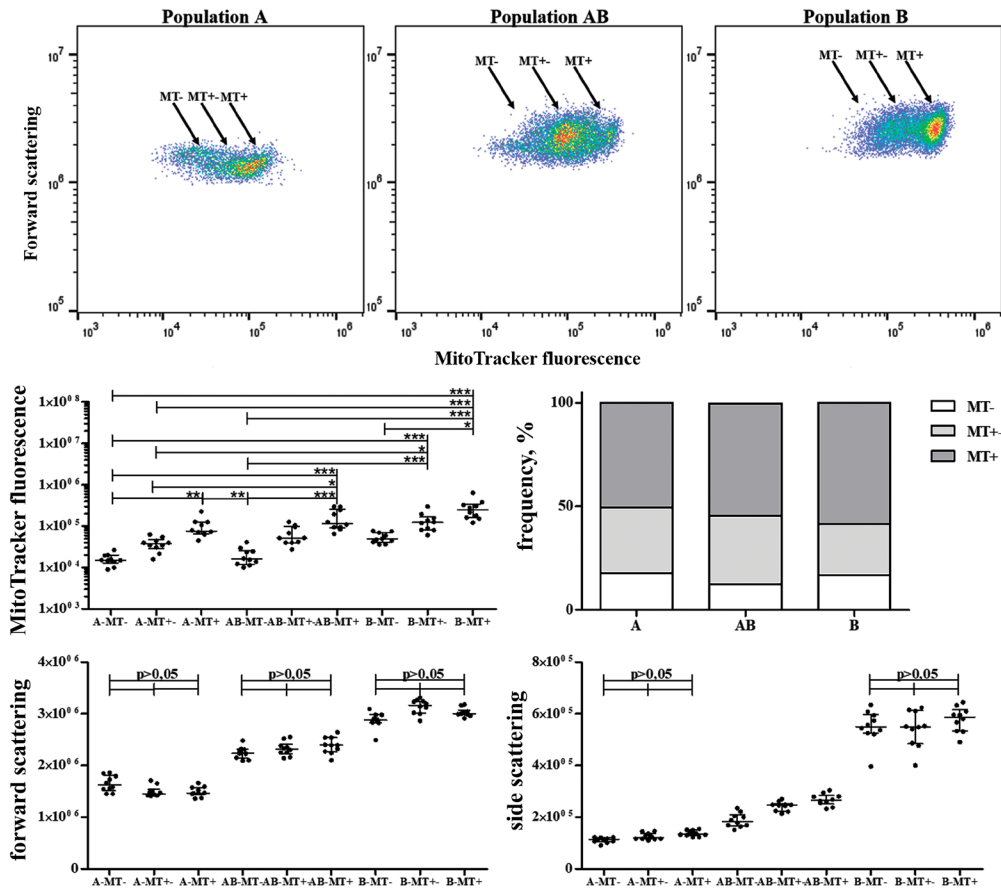


Fig. 6. MitoTracker fluorescence in different populations of hemocytes of *Viviparus viviparus*.

tion A was, on average, 9.25 (8.87; 9.64) μm , that in population AB, 10.90 (10.35; 11.49) μm , and that in population B, 14.22 (13.37; 15.36) μm . Rounded hemocytes and hemocytes with pseudopodia were present in all populations. It is noteworthy that 75.9% $\pm$ 3.72 of hemocytes in population A did not stain with LysoTracker (Fig. 7). In population AB, the lysosomal probe was detected in approximately 50% of the cells (50.80% $\pm$ 9.04), while in population B it was detected in 71.00% $\pm$ 11.61 of the cells. Rounded cells did not contain stained lysosomes, while hemocytes with detected acidic compartments had pseudopodia, although their size and number varied. In population B, hemocytes with pseudopodia but without lysosomes were also occasionally found. LT⁻ and LT⁺ hemocytes of *V. viviparus* did not differ significantly in the area within populations A, AB, and B, while SSC

increased significantly in the series AB-LT⁻ < AB-LT⁺ < B-LT⁻ < B-LT⁺.

Discussion

There are several hypotheses regarding the origin of the cells of molluscan hemolymph. One of them suggests that hemocytes arise from a common pool of prohemocytes localized in specialized hematopoietic organs (Pila *et al.*, 2016; Ataev *et al.*, 2024). The single-lineage hypothesis is based on the fact that the hemolymph contains blast-like (or round-like) cells that are capable of mitosis and subsequent differentiation first into hyalinocytes and then into granulocytes (Sminia *et al.*, 1972; Martins-Souza *et al.*, 2009). However, new data obtained with the use of modern methods support the third hypothesis according to which hemocyte differentiation is a dynamic

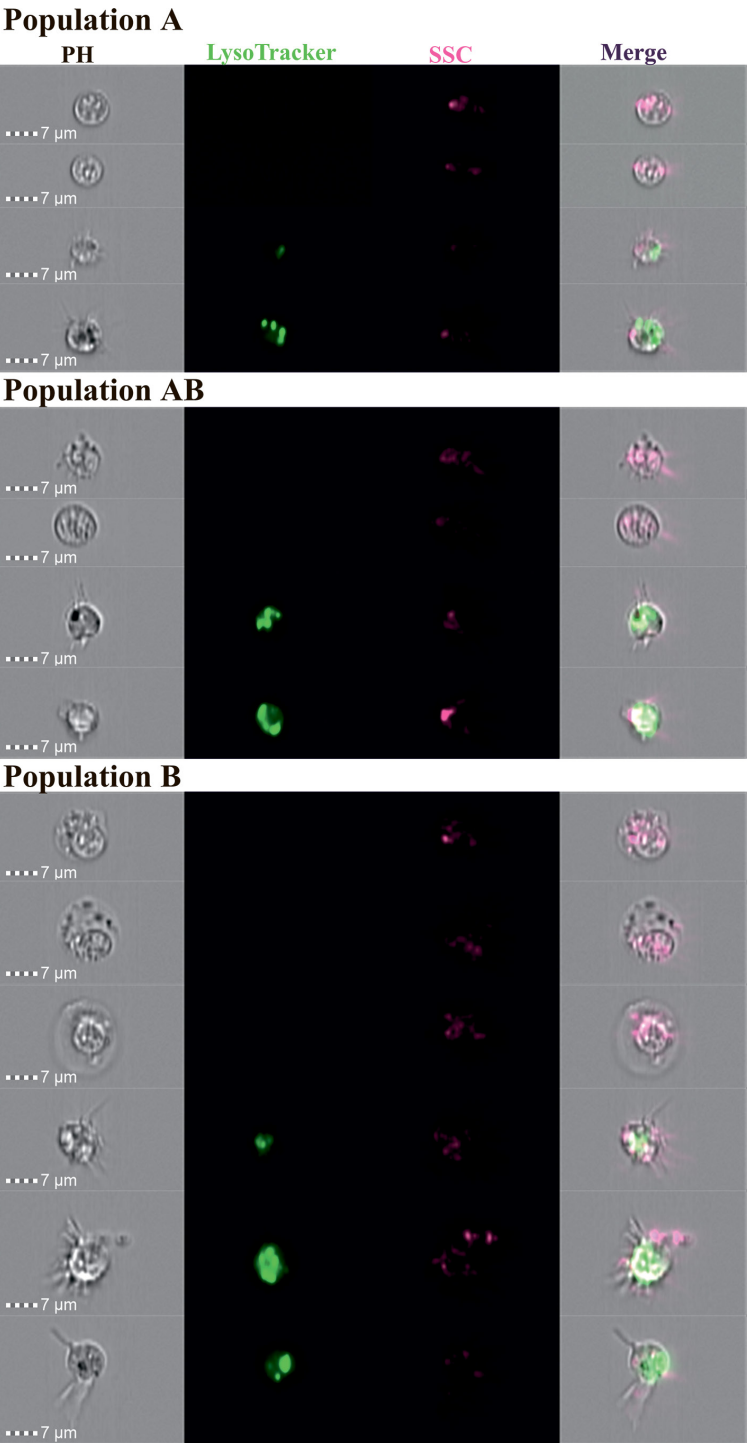


Fig. 7. The analysis of circulating hemocytes in the snail *Viviparus viviparus* performed with the help of Amnis® ImageStream imaging flow cytometer. Accumulation of LysoTracker (green) and SSC (magenta) in hemocytes is shown. PH — phase contrast image of signals characterizing cell morphology.

continuum rather than a strict cellular hierarchy (Pichon *et al.*, 2022). An important argument in its favor is the high plasticity of hemocytes indicating that they are capable of functional reprogramming depending on the immune status of the molluscan organism. At the same time, the final phenotype of cells is determined by ontogenetic and micro-environmental factors.

The controversy concerning the origin of molluscan hemocytes hinders the development of their unified classification. The variety of the proposed classification variants is also due to the fact that different numbers of hemocyte morphotypes has been described in different mollusks. In addition, a standard method for determining cell populations has not been developed yet (Ataev *et al.*, 2024). At present, there are three main typologies of hemolymph cells:

(1) one type of circulating cells — amoebocytes (Sminia *et al.*, 1972; Anderson, Good, 1976; Franchini, Ottaviani, 1990);

(2) two types of circulating cells — hyalinocytes (rounded cells without large pseudopodia) and granulocytes (spreading cells containing granules) (Ottaviani, Montagnani, 1989; Adema *et al.*, 1991; Sasaki *et al.*, 2003; Ataev *et al.*, 2016; Tokmakova *et al.*, 2020);

(3) three types — blast-like cells (small, rounded), hyalinocytes (large, spreading, agranular) and granulocytes (spreading cells containing granules) (Coustau, Yoshino, 1994; Monte *et al.*, 2019; Portet *et al.*, 2019; Serebriakova *et al.*, 2024).

The use of flow cytometry makes it possible to identify the types of circulating hemolymph cells based on FSC and SSC parameters (Granath, Spray, 1987; Accorsi *et al.*, 2013; Grandiosa *et al.*, 2016; Van Nguyen, Alfaro, 2019). For example, two populations of hemocytes were found in *Planorbarius corneus* (Franceschi *et al.*, 1991; Ataev *et al.*, 2016). At the same time, Amen *et al.* (1992) have shown that there is only one population of hemocytes in *Lymnaea stagnalis*, while another research team has identified three groups of hemocytes in these mollusks (Boisseaux *et al.*, 2016). Three populations of hemocytes were also found in other gastropods (Ray *et al.*, 2013; Kajino *et al.*, 2022).

Based on microscopic and cytometric analysis, we identified two types of hemocytes in *P. corneus* and three types of hemocytes in *V. viviparus*. These results confirm our previous

data (Prokhorova *et al.*, 2018a; Serebriakova *et al.*, 2024). Hemocytes of populations A and B in mollusks of both species differ in size, type of pseudopodia, granularity, presence of vacuoles, etc. In addition, we identified cell population C, but the analysis with the use of various methods indicated that it was represented by cells at different stages of apoptosis (Tokmakova *et al.*, 2020; Serebriakova *et al.*, 2024).

ImageStream technology allows the visualization of cell populations detected by classical flow cytometry (Accorsi *et al.*, 2021). However, both rounded cells and cells with pseudopodia were visualized in each population. Therefore, we cannot be sure whether the cells in question belong to a particular morphotype or represent ontogenetic stages of hemocytes of the same morphotype. Unfortunately, there is currently no objective marker for a reliable separation of different hemocyte morphotypes by cytometry. Nevertheless, the use of specific dyes during cytometric analysis has made it possible to identify hemocytes with greater or lesser fluorescence of the LysoTracker lysosomal probe among cells of different populations in both mollusks.

At the same time, ImageStream with the use of LysoTracker revealed a functional difference between rounded cells and cells with pseudopodia. Hemocytes of *P. corneus* and *V. viviparus* that accumulate LysoTracker always have pseudopodia, indicating a high level of activation (Parakhonsky, 2018). At the same time, cells unstained by this fluorescent probe are almost always rounded though they may be different in other morphological characteristics.

To sum up, the presence of acidic granules detected by LysoTracker is a marker of functionally mature granulocytes. At the same time, the presence of lysosome-containing cells in all populations isolated by flow cytometry does not prove that population A is represented only by hyalinocytes while population B is represented only by granulocytes.

Cell populations identified based on LysoTracker accumulation levels are more discrete in *P. corneus* than in *V. viviparus*. For instance, approximately 90% of hemocytes in population A of *P. corneus* have a low LysoTracker fluorescence, while 90% of hemocytes in population B have a high LysoTracker fluorescence. In *V. viviparus*, the ratio of LT⁺/LT⁻ hemocytes is 1:3, 1:1, and 3:1 in populations A, AB, and B, respectively.

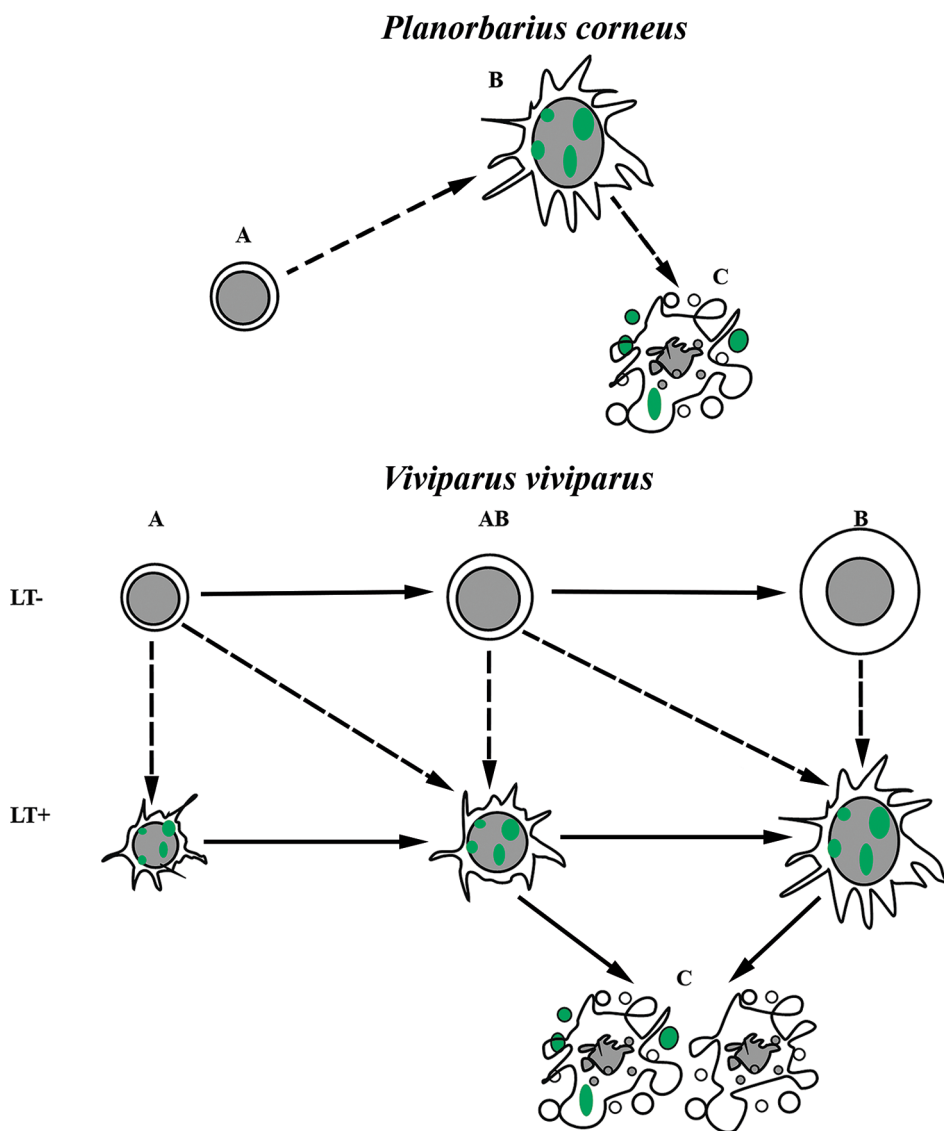


Fig. 8. Hypothetical schemes of hemocyte differentiation in mollusks *Planorbarius corneus* and *Viviparus viviparus*. Solid arrows indicate the process of differentiation of discrete cell lines; broken arrows indicate the process of functional maturation of a single line of hemocytes.

A comparison of the ability of circulating cells to accumulate LysoTracker demonstrates an evolutionarily conservative link between the lysosome activity and the functional state of immune cells (Sharonov, Khramova, 2009; Ballabio, Bonifacino, 2020). In mollusks, as in mammals, cells with a high LysoTracker fluorescence possess pseudopodia and show an

increased phagocytic activity. These features are characteristic of activated neutrophils and macrophages.

The number of active mitochondria is an additional parameter for assessing the specialization and functional maturity of the hemocytes. The membrane potential of mitochondria is an important indicator of their performance and

can be considered as a measure of the intensity of aerobic metabolism (Solaini *et al.*, 2007).

Special fluorescent mitochondrial probes are used in flow cytometry to determine the relative membrane potential of mitochondria (Ly *et al.*, 2003; Solaini *et al.*, 2007). They are commonly used in viability assays, because decreasing mitochondrial potential often forestalls apoptosis (Pedersen, 1999; Ly *et al.*, 2003). However, a decrease in potential can also occur during glycolysis or accompany low levels of oxidative phosphorylation, which is characteristic of stem cells in the resting state (Xu *et al.*, 2013). After the start of differentiation processes the cells switch to a more oxidative phenotype (Chandel *et al.*, 2016). Increased mitochondrial respiration and intracellular ROS levels induce stem cells to enter the cell cycle, and their ability to self-renewal reduces (Maryanovich *et al.*, 2015).

It is also known that immune cells perform their functions using different metabolic pathways. For instance, immature and mature neutrophils have different metabolic requirements, so immature cells mainly rely on fatty acid oxidation, while mature cells prioritize aerobic glycolysis and produce NADPH from PPP (Breda *et al.*, 2019).

A significant variability of the mitochondrial activity in the hemocytes of population A in *P. corneus* suggests the presence of functionally different cells in the hemolymph. At the same time, population B mainly consists of active hemocytes with a high mitochondrial membrane potential. It is interesting that in *V. viviparus* no increase in the ratio of cells with active mitochondria in population B is observed, and all populations consist of cells with various mitochondrial activity.

As secondary messengers, ROS are involved in various physiological processes such as signaling and proliferation during cell adhesion and gene expression (Hamanaka, Chandel, 2010; Noctor, Foyer, 2016). Phagocytes use superoxide radical together with other types of ROS to destroy bacteria (DeLeo *et al.*, 1999). Besides, they actively contribute to the functioning of the immune system by controlling cell proliferation and differentiation (Breda *et al.*, 2019). It is known that non-stimulated hemocytes produce small amounts of hydrogen peroxide (Hambrook *et al.*, 2019) and that granulocytes produce approximately twice as much ROS as hyalinocytes

(Kajino *et al.*, 2022).

At the same time, it should be noted that the hemocytes in different populations are heterogeneous in terms of ROS production. In *P. corneus*, discrete groups of hemocytes differing in this respect were identified in population A (to note, discrete groups were also identified in this population in the analysis of mitochondrial potential). In *V. viviparus*, such discrete groups were found in all populations.

Our data on hemocytes with different metabolic activity may indicate that these cells undergo maturation and differentiation processes. Interestingly, in pulmonate mollusks *P. corneus* these processes occur in small hemocytes belonging to population A, while hemocytes of population B are functionally more homogeneous. In contrast, in prosobranch mollusks *V. viviparus* hemocytes with different levels of metabolic and functional activity are present in all populations, and their ratio remains stable against the background of cell 'growth.' This is obviously due to the pathogenic load associated with gill respiration, which requires a constant influx of new hemocytes into the circulation.

Conclusions

A comparative analysis of the cellular composition of hemolymph in representatives of different subclasses of gastropod mollusks (*Planorbarius corneus* and *Viviparus viviparus*) revealed both similarities and differences in the cellular composition and functional activity of their circulating cells. Both species were shown to have discrete populations of hemocytes differing not only in morphological characteristics but also in metabolic activity.

In addition, cytometric studies revealed the existence of population C in the hemolymph of both molluscan species. However, it does not represent an independent type of hemocytes, but consists mainly of cells at different stages of apoptosis. These data confirmed the results of previous studies (Tokmakova *et al.*, 2020; Serebryakova *et al.*, 2022; Serebriakova *et al.*, 2024).

In *P. corneus*, the main morphological cell types are granulocytes, corresponding to large phagocytic cells with a high lysosome content, and hyalinocytes, which are less differentiated rounded cells. This classification was previously proposed in several studies of the cellular com-

position of the hemolymph of other pulmonate mollusks (Yoshino, Coustau, 2011; Prokhorova *et al.*, 2018b) and is consistent with the results of cytofluorimetric analysis, which showed a significant accumulation of specific dyes LysoTracker and MitoTracker in population B, corresponding to granulocytes (Prokhorova *et al.*, 2018a). Therefore, we can assume the existence of a single cell line in *P. corneus*, in which hematopoietic stem cells differentiate into hyalinocytes and can then transform into granulocytes (Fig. 8).

The presence of three cell populations in *Viviparus viviparus* may be due to the fact that its hemolymph contains cells at different stages of maturation and differentiation (Fig. 8). As a result, a transitional population (AB) of hemocytes is present alongside populations A and B.

The accumulation of the specific lysosomal dye LysoTracker, which correlates with the presence of pseudopodia, was found to be a key marker of functional maturity of hemocytes in both molluscan species. In *P. corneus* the LT⁺ and LT⁻ cell populations are more discrete, indicating a clear division into active and inactive forms, whereas in *V. viviparus* a smooth gradient is observed, emphasizing the dynamism of their immune response.

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