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Morphological and functional characterization of hemocytes in cultivated mussel (*Mytilus galloprovincialis*) and effect of hypoxia on hemocyte parameters

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ABSTRACT

The circulating hemocytes of cultivated marine mussel (*Mytilus galloprovincialis*) were investigated using light microscopy and flow cytometry. In mussels two cell types, granulocytes and agranulocytes, were identified based on the existence of two subpopulations of cells differing by size and granularity level on light-scattered plots. Light microscopic observation confirmed the presence of cells with cytoplasmic granules and cells without granulation in hemolymph of mussels. The main type of cells in hemolymph were agranular cells amounting 78.4 ± 8.9% in mussels. Flow cytometry showed that the agranular hemocytes of the mollusks produce significantly less reactive oxygen species compared to granulocytes. Mussel were exposed for 24 h of hypoxia and immune functions including hemocyte mortality, proliferation and reactive oxygen species (ROS) production were analysed using flow cytometric methods. Granulocyte number was higher at low oxygen concentration than that at normoxia; agranulocytes number decreased, in contrast. The ROS production after hypoxic treatment was decreased compared to normoxia level. No significant changes in hemocyte mortality and proliferation were observed.

1. Introduction

In bivalve mollusks, physiological responses to environmental, anthropogenic and disease factors are mediated by cells circulating in the hemolymph [1]. Hemocytes of bivalves are involved in processes of shell repair, transport of nutrients and internal defense reactions [2]. For commercially cultured species, clear understanding the responses of immune system to natural factors is a question of great importance. The involvement of hemocytes in processes of cellular immunity of bivalves requires their precise classification and morphological and physiological characterization. The most common techniques used for these purposes are flow cytometry and light microscopy [3,4]. Although some authors define 4–6 types of hemocytes depending on species, general classification distinguishes two main groups of cells in hemolymph of bivalves – granulocytes and agranulocytes [1,2,5–8]. Immune function of hemocytes is now considered to be one of the most important for commercially cultured species. It is commonly admitted that granular cells, cytoplasm of which contain granules of various color, size and number play a prominent role in bivalves' cellular immunity. These cells have the ability to phagocytize microbial pathogens, produce superoxide radicals and other reactive oxygen species, contributing to intracellular killing [1,6,9].

The functional role of agranular cells is under debates. In general, agranulocytes (hyalinocytes) are less active in the cellular immune responses, such as phagocytosis or generation of oxidative burst [10,11]. In pearl oyster, *Pinctada fucata*, these cells are involved in tissue repair [12]. Some authors postulated that agranular cells are immature generation of granulocytes, and hemopoiesis in bivalves can occur in circulating hemolymph [8].

In Black sea region, *Mytilus galloprovoncialis* is one of the most intensively cultured bivalves for recent decades. However, cellular composition of hemolymph and physiological responses of hemocytes to environmental factors remain poorly studied. Morphological approach for hemocyte characterization allowed distinguishing 3 cell types in *M. californianus* [13], 2 types in *M. edulis* and *M. galloprovincialis* [2,8,14,15] and 1 type in *M. coruscus* [16]. Some authors divide granulocytes of *M. edulis* [1,17] and *M. galloprovincialis* [2] in 2 and 3 subtypes respectively, according to the number of granules in cytoplasm and their basophilic or eosinophilic color. Flow cytometric observation confirms existence of two main cell types in mussel

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hemolymph [8].

Anthropogenically induced hypoxia is known to be an accelerating problem in coastal marine systems [18]; and understanding of the effects of hypoxia on cultured species is of increasing importance. Black sea mussels, like many other shallow-water mollusks, frequently encounter areas of low dissolved oxygen. Hypoxia provokes a wide range of effects on bivalves. Low dissolved oxygen negatively influences growth, survival rate and delay of metamorphosis in larval and juvenile stages of bivalves [19,20]. In adult mollusks, size increase retard is also observed [21]. Baltic sea species demonstrate changes in their burrowing behavior decreasing the burial depth in hypoxic water [22,23]. At a cellular level, hypoxia may induce autoxidation of respiratory pigments, depresses metabolism, and inhibits immune functions of hemocytes [24–26]. In *Mytilidae* family, the effect of low dissolved oxygen is deeply investigated on tropical and sub-tropical species. Exposure to short-time hypoxia (24–96 h) enhanced hemocyte mortality and negatively influenced immune functions of hemocytes (phagocytic activity and ROS production in hemocytes of green-lipped mussel), *Perna viridis*, and brown mussel, *Perna perna* [11,27]. Long-time (1 month) exposure to low dissolved oxygen decreased physiological parameters, such as clearance rate, respiration rate and growth in *P.viridis* and *Mytilus edulis* [28,29]. However, the rate of negative influence of hypoxia on mussels depends on water temperature [30]. The effects of hypoxia on the representatives of *Mytilidae* family from regions with temperate climate are poorly studied; despite they are intensively cultured there.

In the present study, we characterized hemocytes of cultured Black Sea bivalve, *Mytilus galloprovincialis,* using light microscopy, density centrifugation and flow cytometry in the context of comparative morphological and functional properties. The impact of 24 h hypoxia on hemocyte functions including proliferation, mortality and spontaneous ROS production were investigated.

2. Materials and methods

2.1. Sample collection

Adult mussels (*Mytilus galloprovincialis* Lmk.) (shell length 57.8 ± 1.8 mm, 12.9 ± 2.3 g, n = 40) were obtained from shellfish farm (salt lake Donuzlav, Crimea) during October 2017–November 2017. Mussels were grouped into 15–20 individuals and maintained submerged into 50 L tanks containing aerated sea water (oxygen concentration 7.5–8.0 mg*l^{-1}, salinity 17.8 PSU, pH 8.0, 15 … 18 °C). The level of water salinity and acidity corresponded to natural habitat conditions of mussels in the Black sea region. The acclimation period lasted at least 1 week. Mollusks were daily fed with mixture of microalgae.

2.2. Hypoxia modeling

Hypoxic conditions were reached by bubbling nitrogen in seawater in a reservoir tank with mussels to 0.3 mg L^{-1}. Oxygen concentration was controlled by the oxygen sensor (Ohaus Starter 300 D, USA). Hypoxic oxygen concentrations were maintained in the tank for 24 h and the concentration of oxygen was monitored regularly and kept at constant level throughout the experiment. After 24 h starting the experimentation hemolymph was collected from the mussels as described below.

2.3. Hemolymph sampling

Hemolymph (0.1–0.5 ml) was withdrawn from the adductor muscle with a 25-gauge needle and 2-ml plastic syringe and immediately transferred into individual plastic tubes. All samples were kept on ice to prevent hemocytes clumping. Hemolymph from three to five clams was pooled. After sampling, hemolymph was filtered through 20 μm mesh to eliminate aggregates or large pieces of debris. Then samples were centrifuged at 500 g for 5 min and the pellet was washed twice and resuspended in sterile filtered (0.2 μm) sea water.

2.4. Light microscopy of hemocytes

After the final washing drops of hemocytes pellet were placed on a glass slide and dried for 24 h on air. Smears were fixed and dyed with May Grünwald solution for 3–5 min and then transferred to Giemsa stain for 30 min before being washed in distilled water. Cells were viewed on light microscope (Biomed PR-2 Lum) equipped with camera (Levenhuk C NG Series). Approximately 1000 cells per smear were examined. For each hemocyte the largest cellular and nuclear diameter has been measured. All morphometric measurements were performed in ImageJ 1.44 p.

2.5. Flow cytometry

For all flow cytometry measurements hemocyte concentration in sterile filtered sea water was adjusted to 1–2 × 10^6 cell ml^{-1}. Suspensions were analysed by a FC500 flow cytometer (Beckman Counter) equipped with an air-cooled argon laser, providing a laser excitation at 488 nm. An FSC threshold was defined in order to eliminate cell debris and bacteria and 50 000 events were counted for each sample. For cell types determination suspensions were dyed with DNA-binding fluorochrome SYBR Green I (final concentration in the probe 10 μM). Hemocytes were readily differentiated from other particles in the hemolymph by the level of SYBR Green I fluorescence. Results are expressed as cell cytograms indicating the size (FSC value), the complexity (SSC value) and the level of fluorescence using the FL1 and FL4 channel.

2.5.1. Intracellular ROS production

To test reactive oxygen species (ROS) production by hemocytes 2-7-dichlorofluorescein-diacetate (DCF-DA) was employed as a fluorescent indicator of oxidative burst. Working solution of DCF-DA was prepared by dilution of the dye in DMSO and kept frozen (−20 °C). The dye is oxidized to highly fluorescent dichlorofluorescein (DCF) in hemocyte cytoplasm and the level of fluorescence represents the capacity of cells to produce ROS. 1 ml of hemocyte suspensions was incubated with 10 μl of DCF-DA solution for 30 min in the dark. The green fluorescence produced by DCF was measured by the FL1 detector of the flow cytometer.

2.5.2. Hemocytes proliferation

DNA content in hemocytes was measured on single-parameter histograms of SYBR Green I fluorescence in FL1-channel. The number of proliferating cells was estimated using standard cell cycle analysis, by the number of cells in S–, G_2-and M-stages. Discrimination of aggregates was performed on two-parameter plots of SYBR Green I fluorescence (amplitude versus width of the signal [31]).

2.5.3. Hemocytes mortality

Hemocytes mortality was determined using propidium iodide (PI). 10 μl of 200 μg ml^{-1} PI solution (Sigma Aldrich) was added to 1 ml hemocyte suspension, and the sample was incubated in the dark for 30 min at 4 °C before flow cytometry analysis. The percentage of dead hemocytes relative to the total number of hemocytes was evaluated on the histograms of PI fluorescence in the channel FL4 of cytometer.

2.6. Percoll centrifugation

Hemocyte subpopulations of mussels were separated by isopycnic centrifugation in a discontinuous Percoll gradient according to the protocol used for molluskan hemocytes [32]. 0.5 ml of hemocytes suspensions was layered over a 1.5 ml of discontinuous gradient and centrifuged at 400 g for 10 min in centrifuge Elmi CM-80 (Russia). Cells concentrated at each layer were collected separately, diluted in sterile filtered sea water and gently washed twice to remove Percoll. The pellet was divided for flow cytometric analysis and preparation of slides. Slides were dyed and observed as previously described (see p.2.4).

2.7. Data analysis

Data were checked for normality with the Shapiroe-Wilk's test. One-way analysis of variance (ANOVA) and Tukey's test were carried out to compare the characteristics of different hemocyte subpopulations observed under light microscope, and the significance of the differences between mean values of flow cytometry data was estimated using student's T –tests. Differences were considered significant at $p \leq 0.05$. The results are expressed as the means and standard errors.

3. Results

3.1. Light microscopy

In mussels two hemocyte populations could be recognized on slides: agranulocytes, and granulocytes (Fig. 1B). Agranulocytes in mussels possessed round shape, large basophilic nucleus with rough structure and narrow cytoplasm surrounding it. Agranulocytes mostly did not form pseudopodia (Fig. 1 AG1-AG3), however, we observed rare agranular cells with 1–2 pseudopodia (Fig. 1-AG4). Morphometric parameters of hemocytes are presented in Table 1. Granulocytes were relatively large, possessed ameboid shape with small eccentric nuclei containing mostly heterochromatin, cytoplasm contained granules. The number of granules varied in the range 2–27. The majority of granulocytes possessed basophilic granules, but eosinophilic granules and mixed color were also presented. In some cases, granulocytes did not possess pseudopodia.

Despite average diameter of agranulocytes and granulocytes significantly differed ($p < 0.05$), in hemolymph of mussels we observed rare agranular cells with diameter close to granulocytes. Similarly, some granulocytes were comparably small to agranulocytes.

3.2. Flow cytometry patterns

Suspensions of hemolymph samples were plotted by forward scatter (FSC) and side scatter (SSC). SYBR Green I positive cells, revealed a single peak of green fluorescence, were considered as the hemocyte population. Variability between hemolymph samples by arbitrary size and granularity within was negligible. Hemocytes of mussels were relatively homogenous by DNA content (Fig. 2A). Proliferation of hemocytes was observed in two probes of six and the number of cells undergoing division was less than 8.5% (Fig. 2B). The diploid peak was characterized with low CV (9 ± 0.8%). The percentage of hemocytes found to be nonviable was low in all hemolymph samples (≤10%).

Two groupings were found in the hemolymph of *M. galloprovincialis* (Fig. 1B). Subpopulation 1, comprised small cells with relatively homogenous cytoplasm. Cells were characterized with close arbitrary diameters (according to FSC value) and wide range of granularity. Cells with low granularity formed the majority in the suspension of hemolymph (78.4 ± 8.9%). Subpopulation 2 amounted 21.6 ± 9.0% of total cell count and was formed by large cells with high SSC value. The cells in subpopulation 2 were heterogeneous by their FSC and SSC values.

3.3. ROS production

All cells in suspension of mussel's hemolymph were characterized with pronounced fluorescence of DCF-DA (Fig. 3). Granulocytes showed level of the dye fluorescence 2 times higher than agranulocytes, which only slightly exhibited fluorescence (Table 2).

3.4. Percoll density gradient

Density gradient centrifugation allowed distinguishing two layers of cells in mussels. The lowest layer (cells with larger density) contained mainly granulocytes and small number of large agranulocytes. These cells formed the cloud of subpopulation 2 in flow cytometry dot plots FS/SS (Fig. 4). Agranular cells observed in this layer always have enlarged diameter comparing to average value for this cell type. The top layer was formed by cells with the lowest density, agranulocytes cells. This layer also contained few small granulocytes with less than 7 granules in cytoplasm. Flow cytometric analysis shown that subpopulation 1 is mainly formed by these cells.

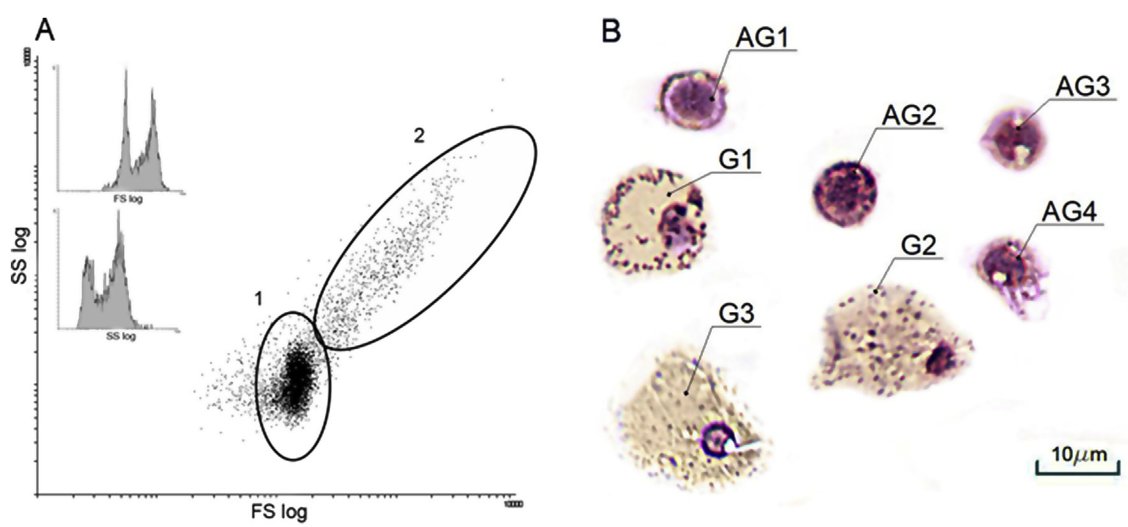

Fig. 1. *M. galloprovincialis* hemocytes. A - Forward scatter (FSC) vs. side scatter (SSC) density plot showing SYBR Green I– positive two hemocyte populations (1 and 2) of mussels' hemolymph. Hemocyte concentration in sterile filtered sea water was adjusted to 1–2 × 10^6 cell ml^{-1}; to measure DNA content cells were incubated with SYBR Green I with final concentration in the probe 10 μM. B - Light microphotographs of hemocytes: AG1-AG4 – agranulocytes; G1-G3 – granulocytes. Slides were stained with May Grünwald solution and viewed in light microscope. Morphometric analysis was performed in the ImageJ program. Bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Dimensions of *M. galloprovincialis* hemocytes measured in slides. Mean ± SE and rank of variation corresponding to cell diameter, nucleus diameter and N/C ratio of each hemocyte type are shown. AG: agranulocyte; G: granulocyte.

	Nucleus (μm)			Cell (μm)			N/C ratio		
	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max
AG	5.4 ± 0.1	2.5	9.5	8.0 ± 0.1	5.3	11.8	0.7 ± 0.01	0.4	1.0
G	5.5 ± 0.2	3.7	7.1	12.7 ± 0.4	8.5	17.7	0.5 ± 0.02	0.2	0.5

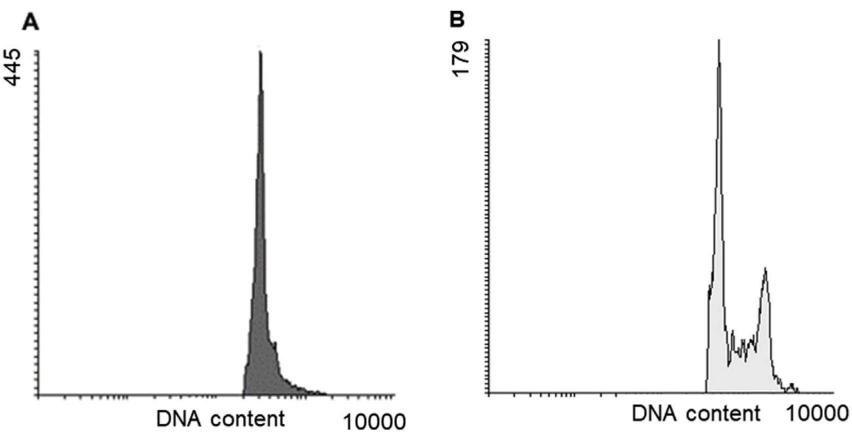

Fig. 2. DNA content in hemocytes of *M. galloprovincialis*. A - histogram showing SYBR Green I-positive non-proliferating cells in suspensions of hemocytes. Single peak of the DNA content represents population of hemocytes. B – proliferating hemocytes in suspensions, showing two peaks of cells SYBR Green I fluorescence corresponding to diploid and tetraploid cells. Hemocyte concentration in sterile filtered sea water was adjusted to 1–2 × 10^6 cell ml^{-1}; to measure DNA content cells were incubated with SYBR Green I with final concentration in the probe 10 μM.

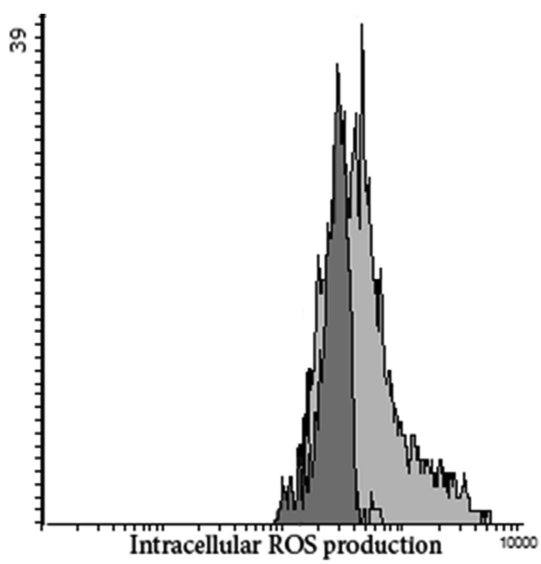

Fig. 3. Intracellular ROS production by hemocytes of *M. galloprovincialis*. Dark grey histogram shows ROS production by agranulocytes, light grey peak corresponds to intracellular ROS content in granulocytes. Hemocyte suspensions were incubated with 10 μl of DCF-DA solution for 30 min in the dark and the fluorescence was viewed in FL1-channel of the flow cytometer. Hemocyte concentration was adjusted to 1–2 × 10^6 cell ml^{-1}.

Table 2
Comparison of the mean DCF-DA fluorescence level (in arbitrary units ± SE) in non-stimulated hemocytes types of *M. galloprovincialis*. * represents significant differences between average value of fluorescence between cell types, *p ≤ 0.05*.

Cell type	DCF-DA fluorescence (arbitraty unit)
Agranulocytes	1216.4 ± 252.1
Granulocytes	2595.7 ± 382.2*

3.5. Hypoxic impact

The cellular composition of hemolymph of mussels, subjected to hypoxia, underwent substantial changes. We observed significant decrease of the agranulocytes number (54.7 ± 8.3% in normoxic mollusks versus 32.3 ± 1.2% for hypoxic probes). The number of granulocytes increased and amounted (56.2 ± 1.0% in hypoxic mussels) (Fig. 5A). No significant changes in the level of dead and proliferating cells were observed. Functional properties of the hemocytes have been modified after hypoxic impact: bidirectional changes in the ability of cells to produce intracellular ROS occurred. In agranulocytes, DCF-DA fluorescence was almost twice greater comparing to the normoxic specimens. To the contrary, the intracellular ROS level in granulocytes of hypoxic mussels significantly decreased (Fig. 5B).

A – cell ratio in hemolymph; B – relative ROS levels after 0.3 O_2 mg l^{-1}. Hypoxia significantly reduced the ROS levels in hemocytes. Error bars represent the standard errors. * with unpaired *t*-test $p < 0.05$ with normoxic mollusks vs. hypoxic specimens.

4. Discussion

The light microscopic and flow cytometric observations of hemocytes from specimens of *M. galloprovincialis* indicate the presence of two different cell types: cells without inclusions in cytoplasm and granulated cells. Granulocytes are larger cells and are able to produce pseudopodia. The results of Percoll density centrifugation showed that cells in hemolymph can be separated by their density. The present study agrees with the previous findings: cell diameter and N/C ratio of hemocytes were close to those obtained by Carballal et al. (1997) [2]. Two general types of hemocytes, agranulocytes and granulocytes have been distinguished in *Mytilidae* family [8,14,33]. Flow cytometric analysis of each layer of cells demonstrated, that granulocytes and agranulocytes form distinct subpopulations in FS/SS dot plots. Granulocytes in mussels can be classified on the basis of color [2], and number and size of granules [34]. In the present work granules of basophilic and eosinophilic color were commonly seen on slides; and cells with intense and poor granulation were also observed. The average diameter of granules also varied among granulocytes. Despite some authors divide subpopulations of granulocytes in representatives of *Mytilidae* family [26],

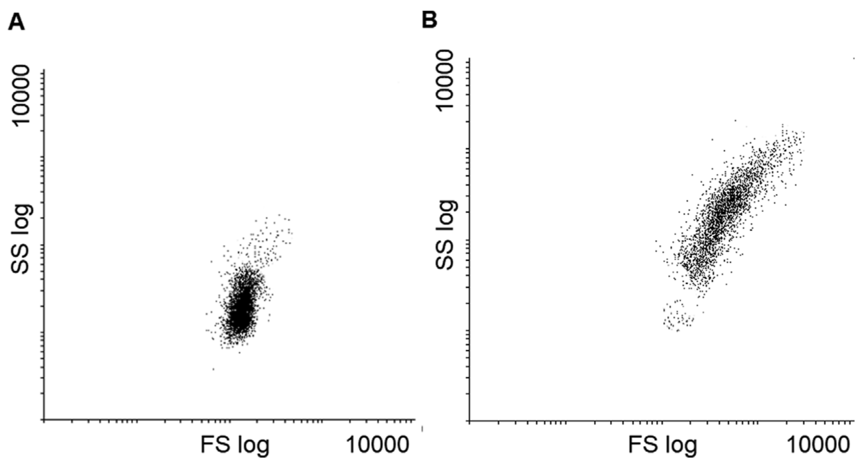

Fig. 4. Flow cytometric analysis of subpopulations of *M. galloprovincialis* hemocytes divided by density gradient centrifugation. A – agranulocytes, forming the top layer in density gradient; B – granulocytes, forming bottom layer of cells with high density. Gradient density centrifugation was performed in Percoll liquid and then washed cells were analysed using flow cytometry technique.

we did not use further identification of hemocyte subtypes as these cells could not be distinguished using flow cytometry or gradient centrifugation and therefore any functional properties of hemocyte subtype could not be studied.

The functional role of each hemocyte subpopulation in bivalves has not been fully studied yet. In *M. galloprovincialis* both granulocytes and hyalinocytes contributed to spontaneous ROS production, but granulocytes seem to be the most active cells. The role of granulocytes in internal defense of mussels may be matched by their capacity for intracellular killing. In *M. edulis* and *M. galloprovincialis* the ROS production have been directly associated with phagocytosis [35,36]. Several observations suggest that granulocytes play a central role in cellular immunity in bivalve mollusks [37,38]. Specific functions for the agranulocytes are not clear. In normal physiological state agranular cells represent the majority of cells in hemolymph [1]. However, these cells aggregate less, produce less superoxide anions than granulocytes and may be non-phagocytic or low-phagocytic [1,38]. Several authors reported that agranular hemocytes are developmental stages of granulocytes [8,39,40]. Agranulocytes of mussels possess larger N/C-ratio than granulocytes. It is known that immature blood cells of lower vertebrates [41] and mammals [42] usually are characterized with high N/C-ratio and basophilic cytoplasm. However, the site of hematopoiesis in bivalves is unknown and hemocytes proliferation is still discussed to be normal or pathological process [1,43,44].

Our results demonstrated that 24 h hypoxic treatment significantly affected hemocyte parameters with immune functions in *M. galloprovincialis*. Low oxygen concentration influenced changes in the cellular composition in hemolymph, which could be an evidence for the hypoxia-induced variations in the whole organism. Agranulocytes number decreased and granulocytes rate significantly rose. Increased granulocyte number in hemolymph agrees with the results of the Pacific oyster *Crassostrea gigas* [45]. This could be unlikely caused by their enhanced proliferation in hemopoietic sites due to relatively short experimentation period. We also did not observe any changes in the rate of dividing cells in suspension. Many authors report the decrease of total hemocyte count in the hemolymph of mollusks underdoing hypoxia [26,46], which is caused by enhanced cellular mortality. In our study, we did not observe increased number of dead cells in the suspensions. Dead hemocytes might be eliminated from the hemolymph by other hemocytes [47]. Moreover, cell mortality depends on the level of species tolerance to hypoxia and the duration of the impact.

Oxygen deficiency also led to low ROS production in granulocytes. DCF-DA fluorescence in agranulocytes increased, in contrast. In controls, ability to spontaneous ROS production in granulocytes was more than twice higher, comparing to agranular cells, presuming their contribution to immune responses of mussels the most significant. It is seemed, that substantial decrease of the ROS concentration in granulocytes induces poor oxidative defense. Changes in DCF-DA fluorescence in hemocytes were probably caused by the inhibition (activation) of some enzymes, which are involved in production (eliminating) of ROS. Many authors reported that hypoxia greatly inhibits the ability of bivalve hemocytes to produce ROS [11,46]. Intracellular ROS production is considered to be proportional to available oxygen concentration as main sources of free radicals within hemocytes are mitochondrion and endoplasmic reticulum [4,45,48]. Experimentation period (24 h) is enough for switching of hemocyte metabolism to anaerobic pathways

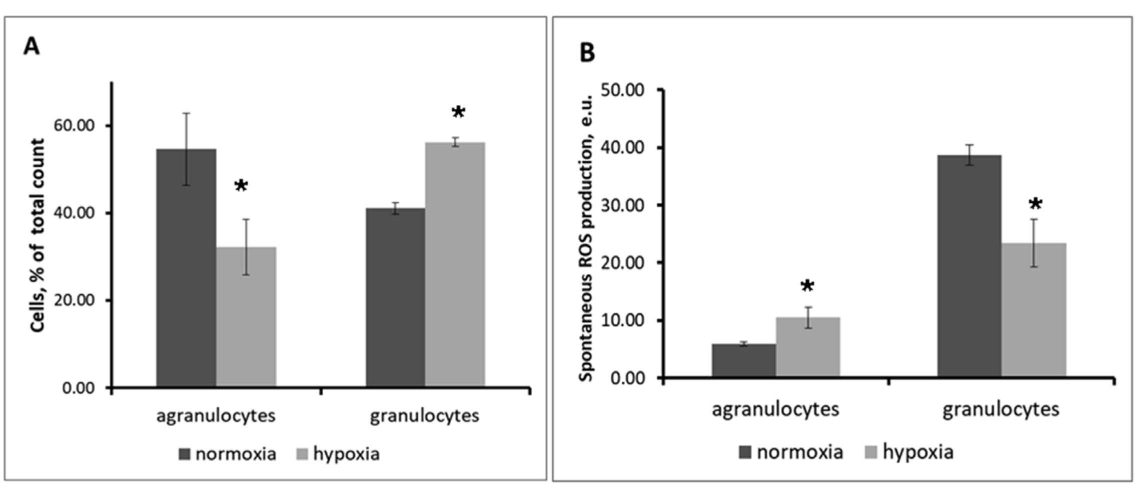

Fig. 5. The response of *M. galloprovincialis* hemocytes to hypoxia.

through a mechanism for HIF-induced adjustments [49]. Unlikely, experimental evidences clearly demonstrating the nature of ROS in hemocytes remain limited. Thus, the actual mechanisms of hypoxic influence on hemocyte immune functions are still unknown.

Thus, flow cytometry, Percoll density centrifugation and light microscopy of *M. galloprovincialis* hemocytes represented similar number of cell types. Hemolymph of *M. galloprovincialis* was mainly composed of small agranulocytes although about 22% of large granulocytes with pseudopodia could be also observed. Hypoxia (24 h) led to substantial increase of granulocytes and the decrease of agranulocytes in hemolymph. Intracellular ROS production in granulocytes was depressed.

Authors contributions

Conceptualization, project administration and methodology performed by A.A.Y. Flow cytometric, visualization and investigation process carried by E.E.S, light microscopic analysis and writing of original draft performed by K.T.A. MS review and editing was made by A.A.Y.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.017.

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