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RESEARCH PAPER

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Functional Characterization of the Pacific Oyster, *Crassostrea gigas* (Bivalvia: Ostreidae), Hemocytes Under Normoxia and Short-Term Hypoxia

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Abstract

Hemolymph cellular composition, morphology and functional properties of the Pacific oyster (*Crassostrea gigas*) hemocytes were studied. Three hemocyte types (agranulocytes, hyalinocytes and granulocytes) were described in hemolymph. The morphology of each type was characterized by light microscopy and flow cytometry. Agranular cells (agranulocytes and hyalinocytes) were the dominant type of cells in hemolymph; their number was 86.7±2.7% of total cell count. Under hypoxia the number of agranulocytes increased (37.4% for control group versus 95.3% for hypoxic probes), whereas granulocyte and hyalinocyte number decreased up to 3.9% and 0.7% in hypoxic specimens respectively. The spontaneous ROS production decreased in each hemocyte type after exposure to hypoxia. Low dissolved oxygen did not influence hemocyte proliferation and mortality level.

Introduction

Hypoxic areas or even persistent oxygen minimum zones (OMZ) (Levin, 2002) has become ubiquitous in coastal and open ocean environments since 1950-ies (Gewin, 2010; Middelburg & Levin, 2009). Coastal hypoxia has expanded in recent decades (Rabalais & Turner, 2001) negatively influencing biodiversity of resident organisms and fisheries. Climate models predict general decrease of oxygen concentration in World Ocean and spreading of OMZ in conditions of global warming (Beszczynska-Möller et al., 2012; Deutsch et al., 2011; Melzner et al., 2013; Chan F. et al. 2019; Zhao et al., 2020). Shelf macroorganisms are usually characterized with low tolerance to hypoxia and up to 50% of coastal species die at oxygen concentration less than 70 mM kg^{-1} (Vaquer-Sunyer & Duarte, 2008; Young & Gobler, 2020).

Due to a prominent ecological role and economic importance of bivalves, the influence of hypoxia on mollusks' physiology has been intensively studied in recent years (van der Schatte Olivier et al., 2020; Gu et al., 2019). Low oxygen induces various physiological disturbances in bivalves, which occurs as a result of decreased protein synthesis and general suppression of aerobic metabolism (Gewin, 2010; Hochachka & Somero, 2002). Hypoxia causes disruptions of growth rate, immunity status and other parameters which are important for cultivated species (Clark et al., 2013).

The Pacific oyster (*Crassostrea gigas*), a commercially important bivalve mollusk, usually inhabit shallow intertidal areas, which frequently became hypoxic due to eutrophication and poor water mixing (Gray et al., 2002; Melzner et al., 2013; Wu, 2002). It is generally considered that species from *Ostreidae* family possess 3 types of hemocytes: agranulocytes, hyalinocytes and granulocytes (Ford et al., 1994; Picot et al., 2019; Hong et al., 2012). Oyster granulocytes are the main hemocyte type involved in cellular immune responses as they are more active in phagocytosis compared with agranulocytes and hyalinocytes (Wang et al., 2017). The hyalinocytes possess lower ability to

phagocytosis, and agranulocytes, in turn, are not able to phagocytoze (Terahara et al., 2006; Takahashi et al., 2017; Jiang et al., 2018). Other bivalve species demonstrate similar specialization of hemocytes (Cheng & Rifkin, 1970; Foley & Cheng, 1975; Wang et al., 2017; Wu et al., 2018; Sui et al., 2016). Despite that, precise functional role of agranular cells is not clear yet, although they also perform cellular immune responses and may be involved in the processes of wound healing (Suzuki & Funakoshi, 1992).

Non-specific immunity parameters are considered to be critically important for assessment of physiological state of cultivated bivalves. Innate immune system of bivalve mollusks is ensured by hemocytes circulating in hemolymph and non-specific tissue humoral factors (Song *et al.*, 2010). Cellular immune responses typically involve phagocytosis and encapsulation of invading agents, and production of cytotoxic products and intracellular reactive oxygen species (ROS) (Cochennec-Laureau et al., 2003; Li et al., 2014; Song et al., 2010; Wang et al., 2018). Low dissolved oxygen greatly influences non-specific immune responses in bivalve mollusks (Wang et al., 2018). Decreased total hemocyte number, intensity of phagocytosis, lower lysosomal content, increased hemocyte mortality and the content of reactive oxygen species were observed in *Perna viridis* and *Mytilus coruscus* under hypoxia (Sui et al., 2016, Wang et al., 2012, 2014; Sussarellu et al., 2012). At the cellular level, hypoxia causes inhibition of aerobic respiration in mitochondria (Sussarellu et al., 2013) and the increase of expression of antioxidant enzymes (Sussarellu et al., 2010). Oxygen is also essential for ROS production, which are involved in the process of pathogen degradation during phagocytosis (Donaghy et al., 2013; Hermes-Lima et al., 2015). 48-h incubation of oysters (*Crassostrea virginica*) in low-oxygen water led to 70 % decrease of ROS production by hemocytes (Boyd & Burnett, 1999). On the other hand, short-term hypoxia did not cause such effects in *C. gigas* (Sussarellu et al., 2012).

Considering that global decrease of dissolved oxygen is an emerging concern for coastal aquaculture the aim of the present work was to examine effects of 24h hypoxia on hemolymph cellular composition and functional parameters of hemocytes of the Pacific oyster (*C. gigas)* cultivated in the Black sea.

Materials and Methods

Maintaining of Mollusks

Adult oysters (*C. gigas*) of both sexes (shell length 94±3.5 mm, 23±4.2 g, n=16) were obtained from shellfish farm (Salt Lake Donuzlav, Crimea) during October 2017–November 2017. Oysters were divided on 2 groups (8 individuals in each group) and kept in 30 L tanks equipped with flowing sea water system (oxygen concentration 7.5 - 8.0 mg×l^{-1}, salinity 17.8 PSU, pH 8.0, 15 - 18ºC) at least 1 week prior to experiment. Water temperature, salinity and acidity corresponded to that observed in natural habitat conditions of oysters in the Black Sea. During the acclimation period oysters were fed daily with mixture of microalgae.

Hypoxia Modeling

Oxygen concentration in the tank with oysters was decreased to 0.2 mg L^{-1} by bubbling of seawater with nitrogen gas. Hypoxic conditions were maintained for 24 h and the concentration of oxygen was monitored regularly (oxygen sensor Ohaus Starter 300 D, USA) and kept at constant level throughout the experiment.

Hemolymph Sampling

After 24 h incubation period 0.5 - 1.0 ml hemolymph was collected from each oyster with a 25-gauge needle and 2-ml plastic syringe. Hemolymph from individual oysters was analyzed separately. After sampling, hemolymph was filtered to 20 µm mesh to eliminate aggregates or large pieces of debris and cells were separated by centrifugation (500g, 5 min). The pellet was washed twice and resuspended in sterile filtered (0.2 µm) sea water. To prevent hemocyte clumping, all manipulations with samples were held on ice. After the final washing slides were prepared.

Light Microscopy of Hemocytes

Slides were dried on air at least 24 h and then fixed and dyed with May Grünwald and Giemsa stain solutions. Hemocytes were viewed on light microscope (Biomed PR-2 Lum) equipped with camera (Levenhuk C NG Series). Approximately 1000 cells per smear were examined. Morphometric analysis was performed using ImageJ 1.44 p. For each hemocyte the largest cellular and nuclear diameter has been measured. The nucleo-cytoplasmic ratio was calculated using the equation:

N/C ratio = nucleus diameter / cell diameter

Flow Cytometry

For flow cytometry analysis, hemocyte concentration in the suspension adjusted to 1-2×10^6 cell ml^{-1}. Cells were analyzed on 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. Suspensions were dyed with DNA-binding fluorochrome SYBR Green I (final concentration in the probe 10 µM), and hemocytes were readily differentiated from other particles in the hemolymph on the basis of the dye fluorescence. Results are expressed as cell cytograms indicating the size (FSC value), the granularity (SSC value) and the level of fluorescence using the corresponding channel of fluorescence.

Intracellular ROS Production

The production of reactive oxygen species (ROS) by hemocytes was assessed using 2-7-dichlorofluorescein-diacetate (DCF-DA). Working solution of DCF-DA was prepared by the dilution of the dye in DMSO and kept frozen (-20ºC). Hemocyte suspensions (1 ml) were incubated with 10 µl of DCF-DA solution for 30 min in the dark. During staining, the dye was oxidized to highly fluorescent dichlorofluorescein (DCF) in hemocyte cytoplasm. The level of fluorescence represents the capacity of cells to generate ROS. The green fluorescence produced by DCF was registered by the FL1 detector.

Hemocyte Proliferation

DNA content and hemocyte proliferation were determined on a 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-, G2- and M-stages (Nunez, 2001). Cell aggregates were discriminated on amplitude versus width plots of SYBR Green I fluorescence.

Mortality Level

The number of dead cells in hemolymph was investigated by the analysis of the fluorescence of hemocytes stained with Propidium iodide (PI). 10 µl of 200 µg ml^{-1} PI stock solution (Sigma Aldrich) was added to 1 ml hemocyte suspension, and the cells were incubated in the dark for 30 min at 4º C. The percentage of dead hemocytes was evaluated on the histograms of PI fluorescence in the channel FL4 of cytometer.

Percoll Centrifugation

To characterize each hemocyte subpopulation, cells were separated by centrifugation in a discontinuous Percoll gradient according to the protocol used for molluskan hemocytes (López et al., 1997). 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 from each layer were collected separately and gently washed twice in sterile filtered sea water to remove Percoll. The pellet was divided for flow cytometric and light microscopic analysis (see 2.4). Each layer was analyzed separately.

Data Analysis

All flow cytometric measurements were performed at least at 3 replicates. One-way analysis of variance (ANOVA) and Tukey's test were carried out to compare the means. Differences were considered significant at P≤0.05. The results are expressed as the means and standard errors.

Results

No mortality of oysters was observed during acclimation period and 24h hypoxia exposure. Total survival of the animals was 100%.

Light Microscopy

Microscopic observation allowed distinguishing three cell types in hemolymph of oysters: agranulocytes, hyalinocytes, and granulocytes (Figure 1). Agranulocytes were round cells with large rough basophilic nucleus and narrow cytoplasm. Agranulocytes did not form pseudopodia. Relatively large granulocytes possessed ameboid shape, cytoplasm had granules (basophilic, eosinophilic or mixed color); small eccentric nuclei contained heterochromatin. Hyalinocytes were characterized with intermediate cellular diameter and their morphology was similar to that in granulocytes, however, basophilic nuclei were situated in the center of cell and basophilic cytoplasm did not contain granules. Morphometric parameters of hemocytes are presented in Table 1.

Despite the average diameter of agranulocytes, hyalinocytes and granulocytes significantly differed ($P < 0.05$, n = 16), we also observed agranular cells with cellular diameter close to that for granulocytes. Similarly, some granulocytes were almost the same size as agranulocytes. The diameter of hyalinocytes varied in the wide range partly overlapping agranulocytes and granulocytes dimensions.

Flow Cytometry

Hemolymph samples stained with SYBR Green I were plotted by forward scatter (FSC) and side scatter (SSC). SYBR Green I positive cells revealing a single peak of green fluorescence were considered as the hemocyte population. Hemocytes were relatively homogenous by DNA content. No proliferation was observed. The CV of diploid peak was 15.7±0.7 (Figure 2). The percentage of hemocytes found to be nonviable were low in all hemolymph samples (≤10 %).

Three cell populations differing by size and granulation could be detected in *C. gigas* hemolymph (Figure 3). Subpopulation 1 consisted of small non-granulated cells amounting 24.3±2.7% of total cell count. Subpopulation 2 comprised large cells with moderate level of granularity (SSC). These cells were the most abundant in hemolymph (62.4±2.7% of total cell count). Cells in the subpopulation 2 greatly varied by their FSC and SSC level. Subpopulation 3 was the smallest in number (13.2±1.7%) and contained the largest cells with the highest granularity level.

ROS Production

All cells in suspension exhibited bright fluorescence of DCF-DA differing by the intensity among

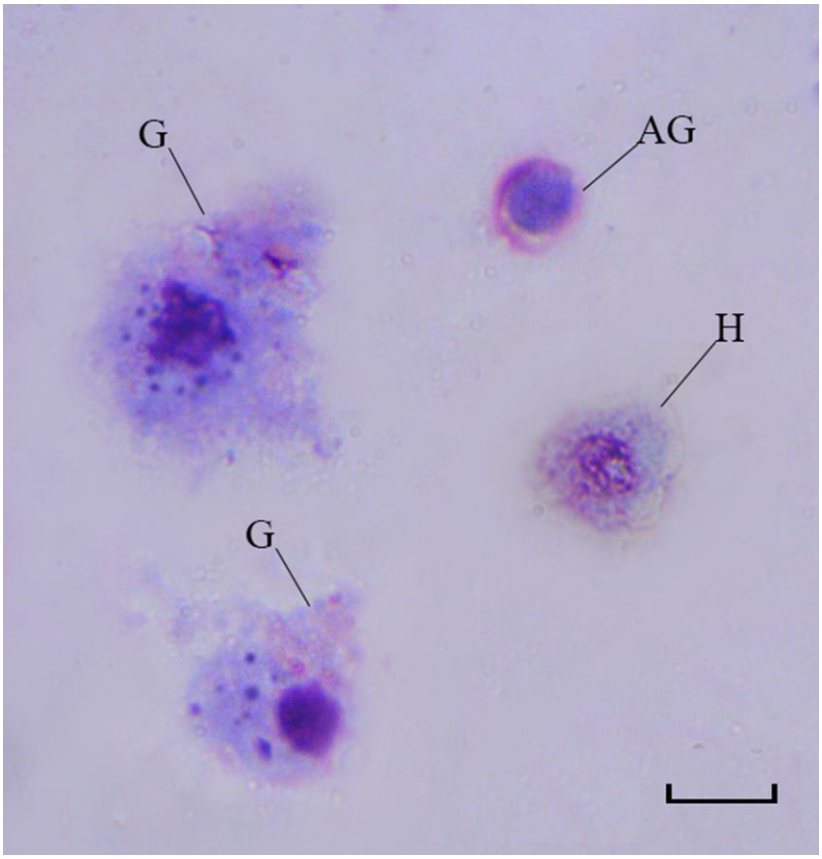

Figure 1. The morphology of *C. gigas* hemocytes: AG– agranulocytes; H-hyalinocytes; G– granulocytes. Slides were stained with May Grünwald and Gimsa solutions and viewed in light microscope. Morphometric analysis was performed in the ImageJ program. Bar: 10µm

Table 1. Morphometric analysis of *C. gigas* hemocytes. Mean ± SE and rank of variation corresponding to cell diameter. nucleus diameter and N/C ratio of each hemocyte type are shown. AG: agranulocyte; H: hyalinocyte; G: granulocyte.

	Nucleus (µm)			Cell (µm)			N/C ratio		
	Mean±SD	Min	Max	Mean±SD	Min	Max	Mean±SD	Min	Max
AG	5.5±0.1	2.4	12.1	9.1±0.1	4.0	20.4	0.6±0.01	0.4	0.9
H	4.0±0.1*	1.2	8.4	9.7±0.2*	4.4	18.2	0.4±0.01*	0.2	0.6
G	3.0±0.1*	1.3	7.6	11.1±0.4*	5.0	23.4	0.3±0.01*	0.1	0.5

* shows a significant differences (P<0.05) between hyalinocyte and agranulocyte types; agranulocyte and granulocyte types.

subpopulations. Subpopulation 1 possessed significantly lower DCF fluorescence compared to subpopulation 2 and subpopulation 3. Subpopulation 2 cells had 3 times higher intensity of the dye fluorescence compared to subpopulation 1. The largest granulated cells were characterized with the highest DCF fluorescence in suspension.

Percoll Density Gradient

Density gradient centrifugation demonsrated three hemocyte layers in hemolymph. The lowest layer (cells with larger density) contained mainly granulocytes and small number of hyalinocytes. Hyalinocytes observed in this layer were larger comparing to mean size for this cell type. The top layer was formed by the cells with the lowest density agranulocytes. Some hyalinocytes were also observed in this layer. The intermediate layer was mainly formed by large agranulocytes and low-granulated granulocytes.

Hypoxic Impact

The cellular composition of oysters' hemolymph. subjected to hypoxia underwent substantial changes. Flow cytometric observation demonstrated significant increase of agranulocyte number (37.4% for control group versus 95.3% for hypoxic probes). The number of granulocytes and hyalinocytes decreased to 3.9% and 0.7% in hypoxic specimens respectively. No significant changes in the level of dead cells hemocyte morphology and proliferation of cells were observed. Hypoxia substantially decreased the ability of hemocytes to produce ROS. The level of DCF-DA fluorescence in all subpopulations of cells was significantly lower comparing to normoxic probes (Figure 4).

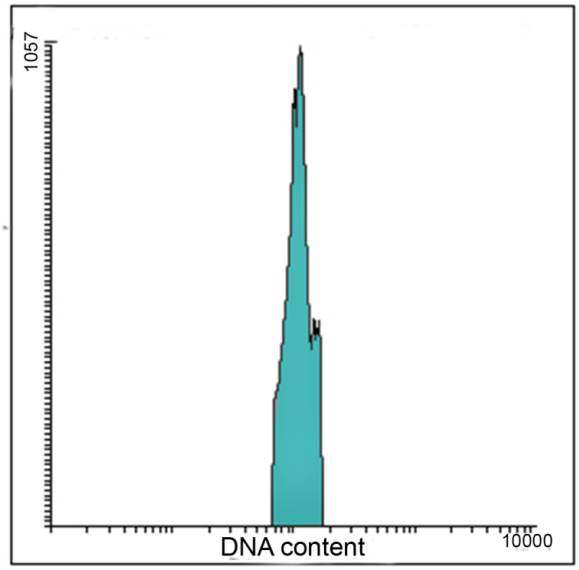

Figure. 2. DNA content in hemocytes of *C. gigas*. Histogram represents SYBR Green I-positive non-proliferating cells in suspensions of hemocytes.

Table 2. Spontaneous ROS production by *C. gigas.* hemocytes. The analysis of ROS production by cells was based on the intensity of DCF-DA fluorescence (Mean ± SE) in non-stimulated hemocytes.

Cell type	DCF-DA fluorescence (arbitrary units)
Subpopulation 1	418±14.11
Subpopulation 2	1290.33±72.67*
Subpopulation 3	5041.89±362.62*

* represents significant differences between average value of fluorescence between subpopulations. P≤0.05

Discussion

Hemocytes Classification

We identified 3 types of *C. gigas* hemocytes that corresponds to classification previously shown by other researchers (Ford et al., 1994; Picot et al., 2019; Hong et al., 2012) The main morphological features of oysters' granulocytes (low N/C ratio. numerous granules in cytoplasm. small eccentric nucleus and pseudopodia) were similar to those already reported (Chang et al., 2005). Some authors subdivided granulocytes into basophilic, eosinophilic and intermediate cells according to the color of granules (Aladaileh et al., 2007; Chang et al., 2005). In spite of observing granulocytes with granules differently colored we did not used this morphological criterion for distinguishing subpopulations as it could be subjective. Density centrifugation of hemolymph demonstrated only three cellular layers and three subsets of cells could be identified in flow cytometry plots, which agrees with previously published data (Bachère et al., 1988). The controversies around the nomenclature of agranular hemocytes in *Ostreidae* family substantially complicate comparison of the cells morphology and functions described in published works. In the present study there were two subtypes of agranular cells in hemolymph, agranulocytes (the smaller ones) and hyalinocytes (the larger agranular cells). These cell types differed by their nucleo-cytoplasmic ratio and the ability to form pseudopodia. Hyalinocytes and granulocytes had similar cellular and nuclear diameters to those found in previous works on *C.gigas* (Ford *et al.*, 1994) and *C.virginica* (Allam et al., 2002). Oysters had larger agranulocytes (9.1 µm *v*. 8.0 µm) and smaller granulocytes (11.1 µm *v*. 12.7 µm) than mussels (Andreyeva et al., 2019). In several works small agranulocytes are sometimes classified as blast-like cells (hemocytoblasts), which are suggested to be stem cells freely circulating in the hemolymph (Hine, 1999). Cells with similar morphology are also called 'small hyalinocytes' (Sun et al., 2006). In the present work agranulocytes had a wide range of cell diameter (4 to 20 µm) and the smallest cells had close diameter and morphology to blast-like hemocytes. Overlap of the range of cell diameter between cell types in the hemolymph observed in this work, and the presence of low- and high- granulated granulocytes in hemolymph may indicate, that agranular cells (i.e. agranulocytes and hyalinocytes) are stages of granulocyte maturation. This hypothesis is indirectly confirmed by the gradual decrease of NCR from agranulocytes to hyalinocytes and granulocytes, as immature vertebrate blood cells usually have larger nucleus compared to mature ones

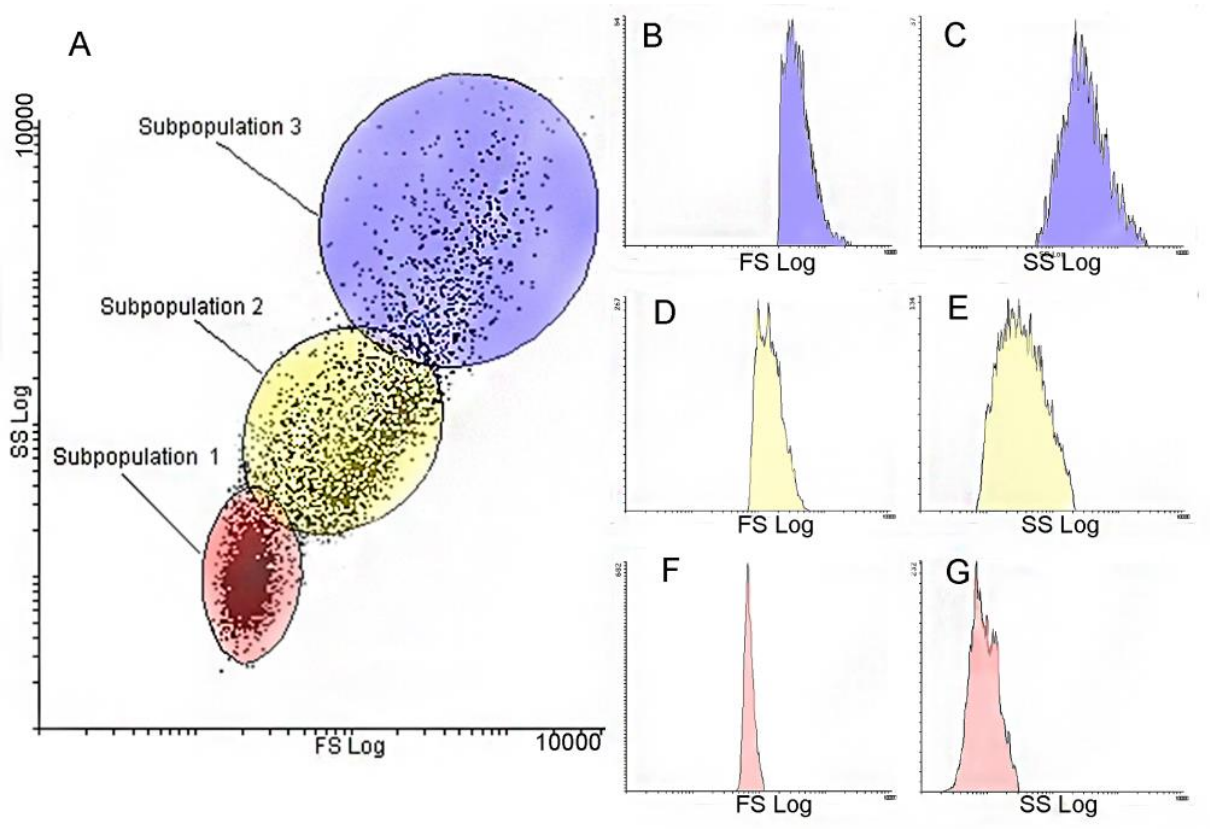

Figure 3. The distribution of *C. gigas* hemocytes by arbitrary size and granulation level. A -Forward scatter (FSC) vs. side scatter (SSC) density plot shows SYBR Green I- positive cells forming three populations (Subpopulation 1.2.3). B. D. F – characterization of cells in subpopulation 3. 2. 1 respectively by the arbitrary diameter; C. E. F – distribution of cells within subpopulation 3. 2. 1 according to granularity criteria. Hemocyte concentration in sterile filtered sea water was 1-2·10^6 cell ml^{-1}; cells were incubated with SYBR Green I with final concentration in the probe 10µM.

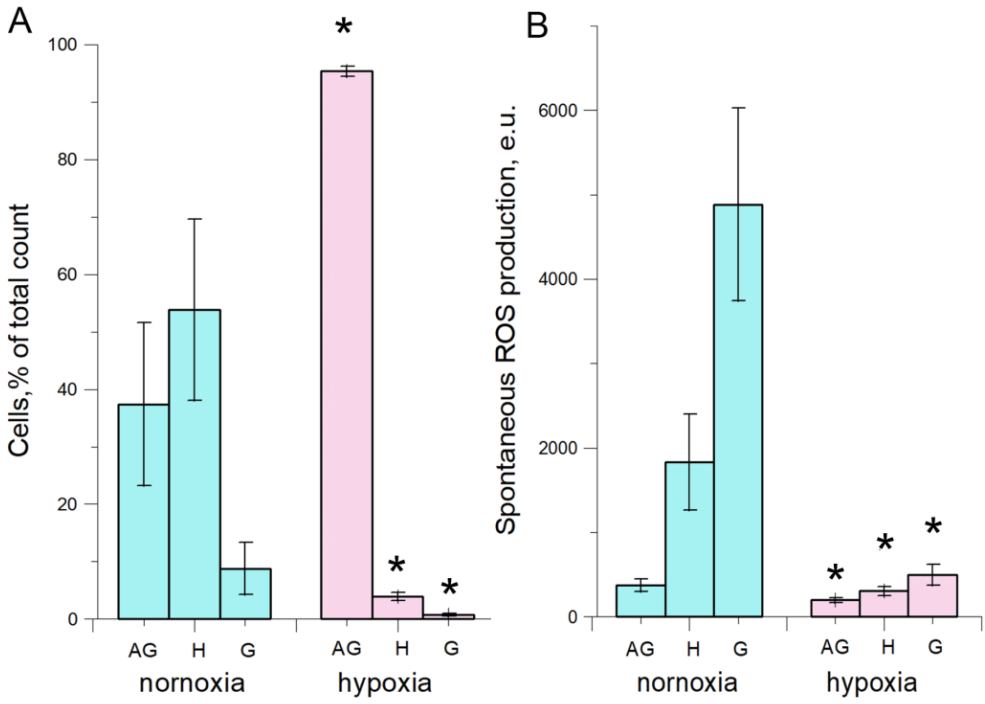

Figure 4. Hypoxia causes changes in cellular composition of *C. gigas* hemolymph and their ability to produce ROS. A – ratio between subpopulations of cells in hemolymph; B – The level of spontaneous ROS production by subpopulations of hemocytes. ROS production by cells was based on the intensity of DCF-DA fluorescence (mean ± SE) in non-stimulated hemocytes. * represents significant differences between average value of fluorescence between cell types. P≤0.05.

(Andreyeva et. al., 2017; van der Knaap et al., 1993). However, site of hemocyte proliferation is supposed to occur outside the circulation system as we did not observe hemocyte proliferation in hemolymph.

Hypoxic Impact

24-h hypoxia substantially influenced functional parameters of hemocytes. Spontaneous ROS production decreased in all cell types. Similar tendency was previously reported for *C.gigas* (Table 2) (Donaghy et al., 2013) and other bivalve species (Andreyeva at al., 2019; Boyd & Burnett. 1999; Wang et al., 2012). Oxygen is essential for production of intracellular ROS and the decrease observed may be at least partly caused by the inhibition of respiration in mitochondria (Donaghy et al., 2012). 24-h hypoxia influenced the agranular and granular cells differently. ROS production in granulocytes decreased more than 9 times. Hyalinocytes and agranulocytes of oysters demonstrated respectively 6 and 2 times lower fluorescence of DCF-DA compared to normoxic probes. Such differences in the degree of hypoxic impact on intracellular ROS production between hemocytes may be caused by the functional role and the morphological structure of each cell type. Granulocytes are more oxygen demanding cells compared to agranular cells, because they possess complicated ultrastructure with numerous mitochondria and endoplasmic reticulum (Chang et al., 2005). These morphological properties presume granulocytes as greater oxygen consumers comparing to agranulocytes which in turn leads to their greater sensitivity to low dissolved oxygen level.

Hemolymph of oysters held in hypoxic conditions contained increased number of agranulocytes. The number of hyalinocytes and granulocytes decreased. Similar changes in agranulocyte number have been previously reported for *C. gigas* after 24-h hypoxia at oxygen concentrations 2.6 mg O_2 L^{-1} (Sussarellu et al., 2012). The processes involved in modulation of hemolymph cellular composition in bivalves under hypoxia are not clear. In lower vertebrates rapid increase of red blood cell number in circulating blood after exposure to hypoxia is usually caused by release of cells from spleen (Fange & Nilsson, 1985; Houston et al., 1996; Strunjak-Perovic et al.. 2009; Abdel-Tawwab et al., 2019; Soldatov A. A. et al., 2017). Changes in blood cell composition following chronic or long-term hypoxia are associated with the enhancement of erythropoiesis in hematopoietic tissues (Moritz et al., 1997; Soldatov, 2005; Van der Weele & Jeffery, 2019). Increased hematopoiesis seems unlikely due to short-term exposure to hypoxia. In the present work we did not observe hemocyte proliferation in normoxic or hypoxia-treated mollusks. Hemocyte migration from other tissues is an alternative process that may be associated with changes in the hemolymph cell composition during hypoxia (Donaghy et al., 2012; Ottaviani et al., 1998).

In summary, hemolymph of the Pacific oyster contains 3 hemocyte types, two of which are agranular cells (agranulocytes and hyalinocytes) and one is granulocytes. Agranular cells are supposed to be immature granulocytes. However, hematopoiesis of hemocytes does not occur in hemolymph. The results of the study indicate that 24-h hypoxic exposure causes considerable changes in physiology of oysters. At the organismic level the fluctuations in hemolymph cellular composition are observed, i.e. the number of agranulocytes increased. At the cellular level incubation in low dissolved oxygen led to decrease of spontaneous ROS production by hemocytes, but did not induce hemocyte mortality.

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