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IMPACT OF 24-HOUR HYPOXIA ON HEMOCYTE FUNCTIONS OF *ANADARA KAGOSHIMENSIS* (TOKUNAGA, 1906)

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Shellfish farms are usually located in coastal areas, where molluscs can be exposed to hypoxia. Cultivating at low oxygen levels causes general disruptions of growth rate, outbreaks of diseases, and mollusc mortality. Impact of short-term hypoxia on hemocyte functions of ark clam (*Anadara kagoshimensis*) was investigated by flow cytometry. A control group was incubated at 6.7–6.8 mg $O_2 \cdot L^{-1}$, an experimental one – at 0.4–0.5 mg $O_2 \cdot L^{-1}$. Exposition lasted for 24 hours. Hypoxia was created by blowing seawater in shellfish tanks with nitrogen gas. In ark clam hemolymph, 2 groups of hemocytes were identified on the basis of arbitrary size and arbitrary granularity: granulocytes (erythrocytes) and agranulocytes (amebocytes). Erythrocytes were the predominant cell type in *A. kagoshimensis* hemolymph, amounting for more than 90 %. No significant changes in cellular composition of ark clam hemolymph were observed. The production of reactive oxygen species and hemocyte mortality in the experimental group also remained at control level. The results of this work indicate ark clam tolerance to hypoxia.

Keywords: ark clam, marine cultivating, flow cytometry, hematological parameters, reactive oxygen species

Hypoxic zones are widespread on the shelf of marine areas (Dang et al., 2013 ; Diaz & Rosenberg, 2008). Shellfish farms are traditionally located in coastal areas, where they can be exposed to stable or periodic hypoxia. Oxygen deficiency results in disruption of growth rate, outbreaks of diseases on farms, and mass mollusc mortality (Andreyeva et al., 2019 ; De Zwaan et al., 1991 ; Nicholson & Morton, 1998 ; Sussarellu et al., 2013). The latter determines the relevance of the search for cultivation species, being characterized with wide adaptive potential for oxygen deficiency. The main cultivation objects on Black Sea coast are *Mytilus galloprovincialis* Lamarck, 1819 and *Crassostrea gigas* (Thunberg, 1793) (Yakhontova & Dergaleva, 2008).

Physiological parameters, such as filtration intensity, respiration rate, and growth rate, are known to deteriorate when mussels lack oxygen (Wang & Widdows, 1991). Hypoxia has the most detrimental impact on juvenile mussels and oysters, in particular on their growth, dispersal, and survival (Baker and Mann, 1992). This can be explained by the fact that under hypoxia, larvae of these molluscs are forced to reduce energy consumption for the processes of food intake, digestion, and growth, thereby reducing the need for oxygen (Wang et al., 2012 ; Wang & Widdows, 1991).

The bivalve mollusc *Anadara kagoshimensis* (Tokunaga, 1906), belonging to Arcidae family, is considered a promising species for industrial cultivation on Black Sea coast (Yakhontova & Dergaleva, 2008). This is due to ark clam wide habitat, high growth rates, and ability to increase biomass at high stocking density (Yakhontova & Dergaleva, 2008). However, the development of a reproduction technology of cultivation object, being not typical for a region, requires an understanding of its physiological responses to stressful environmental factors, in particular hypoxia.

Physiological state of mollusc can be assessed by the functional state of hemocytes: cells, circulating in hemolymph. Physiological impact of oxygen deficiency is assessed by changes in the ratio of hemocyte types, as well as by indicators of non-specific immune response (production of reactive oxygen species (hereinafter ROS) and phagocytic activity) and hemocyte proliferation (Sussarellu et al., 2013, 2010). There are many studies on the impact of short-term hypoxia on traditional cultivation objects: *M. galloprovincialis* and *C. gigas* (Novitskaya & Soldatov, 2011 ; Sussarellu et al., 2012, 2013, 2010 ; Wu, 2002). As a result of hypoxia, some physiological disorders occur at the cellular level in oysters: intensity of mitochondrial respiration decreases (Sui et al., 2016), and expression of antioxidant enzyme genes increases (Sussarellu et al., 2013). Moreover, cases of cell damage are recorded, in particular hemocytes, circulating in the hemolymph (Hermes-Lima, 2015). The latter may result in suppression of immune functions (Donaghy et al., 2013). At the same time, total number of hemocytes increases, which may be due to their proliferative activity under hypoxia (Sussarellu et al., 2010). In mussels lacking oxygen, number of granulocytes increases, while number of agranulocytes, on the contrary, decreases (Andreyeva et al., 2019). Decreased production of ROS in oyster and mussel hemocytes negatively affects their protective functions (Andreyeva et al., 2019 ; Boyd et al., 1999).

Ark clam *A. kagoshimensis* is considered a species, being tolerant to oxygen deficiency due to hemoglobin in pigmented hemocytes – erythrocytes (Soldatov et al., 2010, 2018). It is able to survive for several weeks under deep hypoxia (Holden et al., 1994 ; Mydlarz et al., 2006). Tolerance for oxygen deficiency is ensured due to ability to efficient anaerobic metabolism (Novitskaya & Soldatov, 2011 ; Cortesi et al., 1992 ; Isani et al., 1986 ; Miyamoto & Iwanaga, 2012). It is known that even under acute hypoxia, ark clam is able to maintain intracellular energy balance at a relatively high level (Cortesi et al., 1992 ; Novitskaya & Soldatov, 2013). Moreover, hypoxia does not lead to lysis of ark clam erythrocytes (Andreyeva et al., 2019 ; Zwaan et al., 1995). However, it was noted that after 3 days of incubation under hypoxia, morphological parameters of erythrocytes change (Wang & Widdows, 1991). The impact of hypoxia on the parameters of *A. kagoshimensis* hemocyte cell immunity is not known so far. Based on the available data, it is impossible to draw a conclusion about the immune status of ark clam hemocytes under hypoxia. At the same time, it is the suppression of immune functions that can lead to mass mollusc mortality (Wang & Widdows, 1991 ; Widdows et al., 1989), which creates problems for shellfish farms.

The aim of this work is to study the impact of 24-hour hypoxia on the functional parameters of hemocytes of the bivalve mollusc *A. kagoshimensis* during *in vivo* experiments.

MATERIAL AND METHODS

Bivalve molluscs *A. kagoshimensis* (shell length (15.2 ± 6.1) mm; weight (30.6 ± 2.8) g; n = 20) were collected in the Sevastopol Bay (Sevastopol) in June 2019. To relieve the stress, caused by catching and transportation, ark clams were kept in tanks with running seawater at the rate of 3–5 L per individual; oxygen concentration was maintained at 6.7–6.8 mg $O_2 \cdot L^{-1}$ for a week. Hypoxia was created by blowing

seawater in shellfish tanks with nitrogen gas, until a concentration of 0.4–0.5 mg $O_2 \cdot L^{-1}$ was reached. Ark clams were kept under hypoxia for 24 hours. The control group ($n = 10$) was maintained at an oxygen concentration of 6.7–6.8 mg $O_2 \cdot L^{-1}$.

Hemolymph was collected with a sterile syringe from the extrapalial space. The remaining cells were resuspended in sterile seawater (hemocyte concentration was from $1 \cdot 10^6$ to $2 \cdot 10^6$ cells$\cdot mL^{-1}$). Hemocytes functional characteristics were analyzed on a Beckman Coulter FC 500 flow cytometer. To identify cell types and to assess the DNA content, the prepared hemocyte suspension was stained with a SYBR Green I DNA dye (final concentration in the sample was 10 μmol; incubation period was 30 minutes in the dark). The DNA content in mollusc hemocytes was analyzed on the basis of histograms of the dye fluorescence distribution in the FL1 channel by the Flowing Software 5.2. The DNA content in the cells is on the abscissa axis of the histogram, and number of cells is on the ordinate axis.

The ability of hemocytes to spontaneously produce ROS was assessed by flow cytometry using fluorescence of the 2',7'-dichlorofluorescein diacetate (DCF-DA) dye: 1 mL of hemocyte suspension was incubated with 10 μL of DCF-DA solution for 30 minutes in the dark. Final dye concentration in the sample was 10 μmol. Dye fluorescence was analyzed in the FL1 channel of the flow cytometer (green area of the spectrum).

Hemocytes mortality rate was determined using the fluorescent dye of propidium iodide (PI). To 1 mL of hemocyte suspension, 10 μL of PI solution (Sigma Aldrich) were added and incubated in the dark for 30 minutes at +4 °C. The ratio of dead hemocytes in total number of hemocytes was estimated by the histograms of PI fluorescence in the FL4 channel (red area of the spectrum).

Protocol calibration for the analysis of the arbitrary cell size was performed by fluorescent microspheres with a diameter of 0.9; 2.0; 4.2; 5.7; and 9.0 μm. Normality of distribution was checked by the Shapiro – Wilk test. The statistical significance of the differences was assessed by the Student's t-test at $p \leq 0.05$. The results are presented as mean values and their errors (mean ± SE).

RESULTS

Fluorescence peak of SYBR Green I dye was heterogeneous in hemocytes; it was characterized by a relatively high coefficient of variation (CV) both under normoxia [(21.6 ± 1.4) %] and hypoxia [(21.3 ± 1.1) %] (Fig. 1). Hemolymph samples analyzed contained small number of dead cells: their ratio in the control and experimental samples did not exceed 1.5 %.

The analysis of cells distribution by forward and side scattering (FS and SS, respectively) allowed identifying two cell subpopulations with different arbitrary size and granularity level (Fig. 2). The differences were statistically significant. Subpopulation 1 was characterized by high values of arbitrary size [(1282.7 ± 89.3) arbitrary units] and granularity [(199.2 ± 21.7) a. u.]. According to the data of protocol calibration with latex microparticles, average cell diameter was 14–15 μm. Probably, the elongation of cell distribution along the SS axis was due to differences in nucleus size and number of granules in the cytoplasm. Subpopulation 2 was heterogeneous by FS and SS values [(181.8 ± 18.4) a. u.] and had relatively low values of arbitrary size [(392.8 ± 36.1) a. u.], which corresponded to average cell diameter of 7–8 μm. The heterogeneity of cell distribution indicates presence of several subtypes of cells among amebocytes, but it is impossible to identify these subtypes using existing methods. Based on hemocytes classification (Dang et al., 2013), subpopulation 1 was identified as erythrocytes, and subpopulation 2 – as amebocytes.

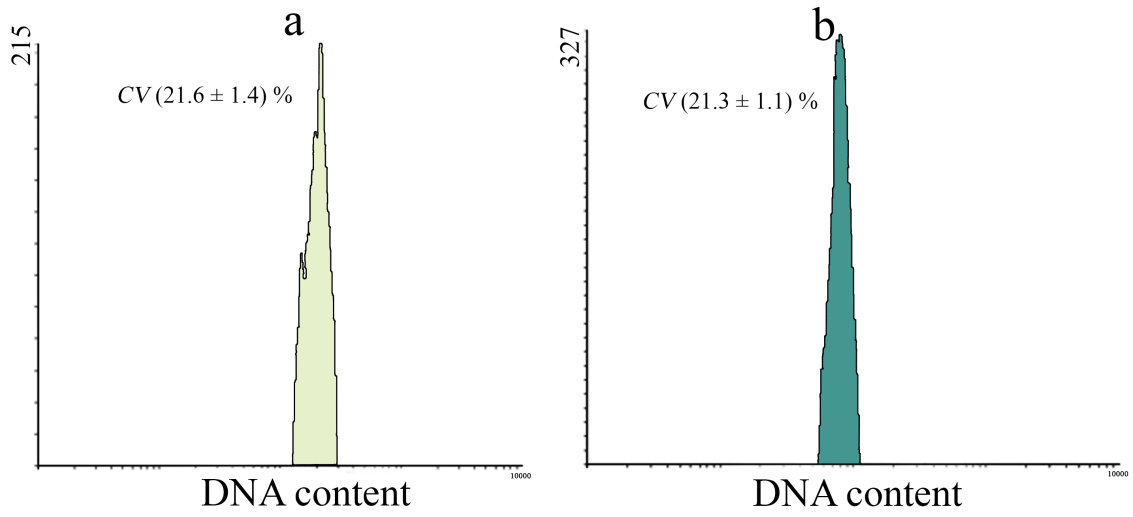

Fig. 1. DNA content in ark clam hemocytes: a – normoxia; b – hypoxia

Cellular composition of *A. kagoshimensis* hemolymph underwent no changes after 24-hour incubation under hypoxia (Fig. 3a). The ratio of granular cells – erythrocytes – in hemolymph of molluscs from the experimental and control groups actually coincided, being (92.6 ± 0.9) and (93.9 ± 1.9) %, respectively. It was similar in relation to agranular cells – amebocytes: (7.4 ± 0.9) % – normoxia; (6.3 ± 1.9) % – hypoxia.

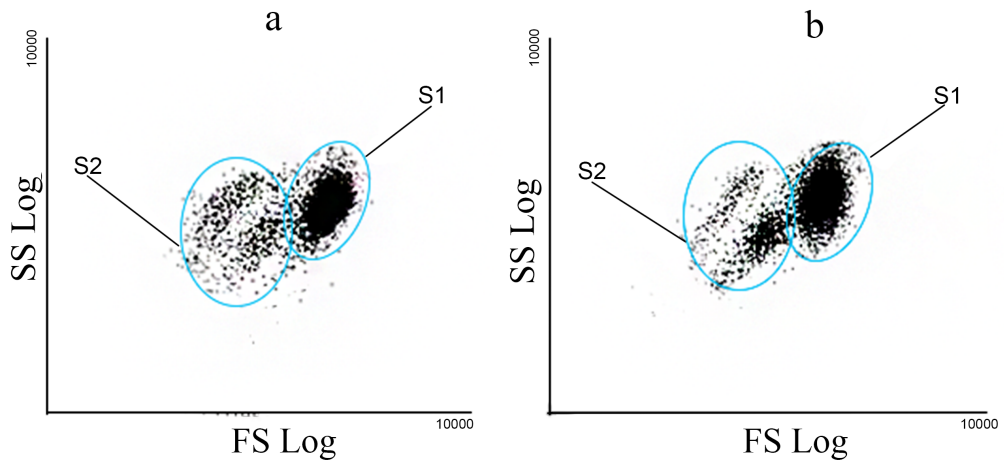

Fig. 2. Cellular composition of ark clam hemolymph. S1 – erythrocytes; S2 – amebocytes; a – normoxia; b – hypoxia

Both cell subpopulations were characterized by pronounced fluorescence of the DCF-DA dye under normoxia: (2439.5 ± 189.0) a. u. for large cells and (4104.3 ± 556.7) a. u. for small ones, which indicates their active production of ROS. Differences in dye fluorescence are not statistically significant. Hypoxia for 24 hours did not lead to statistically significant changes in DCF-DA fluorescence in *A. kagoshimensis* (Fig. 3b).

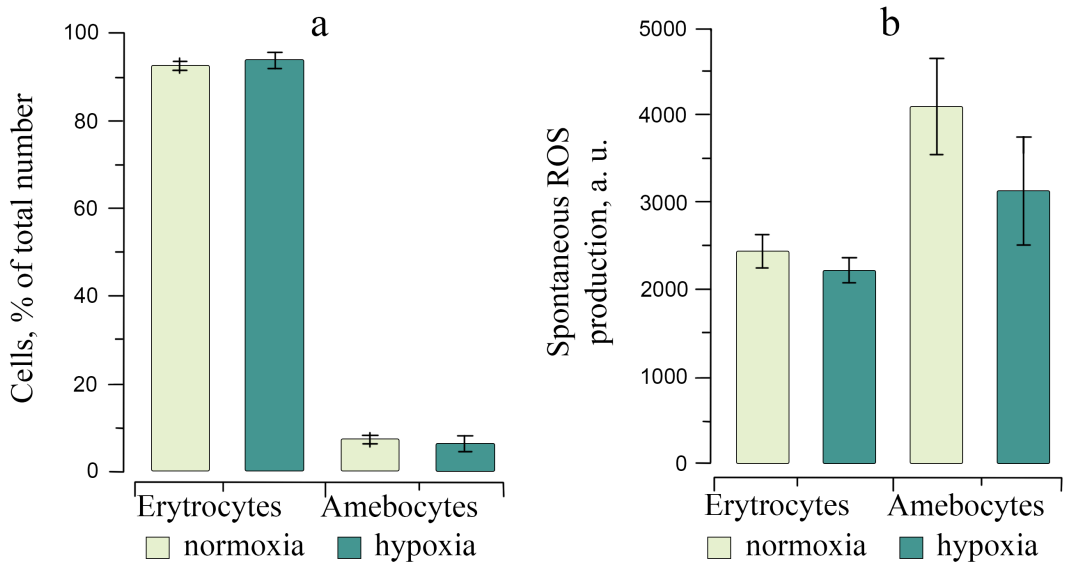

Fig. 3. Hypoxia impact on cellular composition of ark clam hemolymph (a) and hemocytes capacity to produce reactive oxygen species (b)

DISCUSSION

It is known that incubation under oxygen deficiency can induce a decrease in total number of hemocytes (Mydlarz et al., 2006). It was shown as follows: in *Mytilus coruscus* and *Perna viridis*, after incubation under hypoxia, the ratio of dead cells increased (Sui et al., 2016 ; Wang et al., 2012), while in *M. galloprovincialis* their number did not change (Andreyeva et al., 2019). We did not determine absolute number of hemocytes. In our study, hypoxia did not lead to an increase in cell mortality, which probably indicates normal functioning of ark clam hemocytes under short-term hypoxia. The differences can be explained by exposure duration and level of species tolerance to oxygen deficiency.

By flow cytometry, two cell subpopulations with different arbitrary size and granularity level were identified, which, in general, is consistent with data, obtained on other species of bivalve molluscs (Andreyeva et al., 2019 ; Sussarellu et al., 2013 ; Wang et al., 2012). Subpopulation 1 was identified as cells of the granular type – erythrocytes, according to the classification of Dang et al. (2013). Subpopulation 2 was identified as cells of the agranular type – amebocytes (Dang et al., 2013). Both cell subpopulations show pronounced DCF-DA fluorescence, which is not typical for other species of bivalve molluscs (*M. galloprovincialis*, *O. edulis*, and *C. gigas*). It is believed that granular hemocytes are not only responsible for gas transport function, but are also more active, in comparison with agranular ones, in the manifestation of immune reactions: phagocytosis, as well as production of protective peptides and ROS. The generation of an oxidative burst plays an important role in protection against microorganisms, since ROS, in combination with lysosomal enzymes, take part in the phagocytosis reaction, contributing to destruction of opportunistic objects (Sussarellu et al., 2013). The absence of differences in the ability to produce ROS between agranular and granular cells may indicate the absence of functional differentiation of ark clam hemocytes. The latter is consistent with data of previous studies on representatives of Arcidae family (Dang et al., 2013).

The ability to produce ROS directly depends on oxygen content in the environment, since the main source of free radicals is mitochondria and endoplasmic reticulum (Wang et al., 2012). Many authors believe that hypoxia causes a decrease in the ability to produce ROS in other species of bivalve molluscs,

suppressing oxidative protection (Sui et al., 2016 ; Widdows et al., 1989). The mechanism, underlying the maintenance of production of ROS by hemocytes under oxygen deficiency, is not fully understood. It is believed that hypoxia can induce oxidation-reduction changes at the level of electron carriers, which leads to the generation of an oxidative burst at the level of mitochondrial complex III (Chandel et al., 2000). Hemoglobin can participate in this (Jiang et al., 2007). It was shown that after deoxygenation, hemoglobin acquires pseudoperoxidase activity, which can catalyze production of superoxide ion (Kawano et al., 2002). Consequently, peroxidase activity of ark clam hemoglobin can induce production of ROS (Bao et al., 2016), maintaining the ability to immune response under hypoxia. In our case, 24-hour hypoxia does not induce changes in DCF-DA fluorescence in *A. kagoshimensis*. The absence of these changes in our work may indicate sufficient compensatory mechanisms of ark clam to maintain the normal functional state of the endoplasmic reticulum and mitochondria under 24-hour hypoxia.

Conclusion. By flow cytometry, two groups of cells were identified in *Anadara kagoshimensis* hemolymph: granulocytes (represented by erythrocytes) and agranulocytes (amebocytes). The ratio of granulocytes in ark clam hemolymph exceeds 90 % of total number of cells. Hypoxia for 24 hours does not lead to changes in cellular composition of ark clam hemolymph. The ability to produce reactive oxygen species and the mortality rate of hemocytes in molluscs of the experimental group remain at the level of control values (normoxia). The results of this study indicate *A. kagoshimensis* tolerance to acute oxygen deficiency.

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ВЛИЯНИЕ СУТОЧНОЙ ГИПОКСИИ НА ФУНКЦИОНАЛЬНЫЕ ПОКАЗАТЕЛИ ГЕМОЦИТОВ *ANADARA KAGOSHIMENSIS* (TOKUNAGA, 1906)

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Марикультурные хозяйства традиционно расположены в прибрежных участках, где моллюски могут подвергаться воздействию гипоксии. Культивирование в условиях дефицита кислорода приводит к снижению темпов роста, вспышкам заболеваний на фермах и массовой гибели моллюсков. Методом проточной цитометрии исследовано влияние краткосрочной гипоксии

на функциональные показатели гемоцитов анадары (*Anadara kagoshimensis*). Контрольную группу содержали при 6,7–6,8 мг $O_2 \cdot л^{-1}$, опытную — при 0,4–0,5 мг $O_2 \cdot л^{-1}$. Экспозиция — 24 часа. Содержание кислорода в воде снижали, продувая её газообразным азотом. В гемолимфе моллюска на основании относительного размера и относительной гранулярности идентифицировано две группы гемоцитов: гранулоциты (эритроциты) и агранулоциты (амёбоциты). Эритроциты — преобладающий тип клеток в гемолимфе *A. kagoshimensis*: их доля составила более 90 % от общего числа клеток. Суточная гипоксия не привела к изменениям клеточного состава гемолимфы анадары. Способность к продукции активных форм кислорода и уровень смертности гемоцитов моллюсков экспериментальной группы также остались на уровне контрольных значений. Результаты проведённого исследования свидетельствуют о толерантности анадары к условиям острого дефицита кислорода.

Ключевые слова: анадара, марикультура, проточная цитометрия, гематологические показатели, активные формы кислорода