ISSN 1063-0740, Russian Journal of Marine Biology, 2015, Vol. 41, No. 4, pp. 315–320. © Pleiades Publishing, Ltd., 2015.
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ECOLOGICAL PHYSIOLOGY

Erythropoiesis and the Contents of Abnormal Erythroid Forms in the Blood of the Round Goby, *Neogobius melanostomus* Pallas, 1811 (Osteichthyes: Gobiidae)

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Received November 27, 2014

Abstract—The relationship between the contents of abnormal erythroid forms (dacryocytes, nuclear invagination, and erythrocyte ghosts) and the number of immature erythrocytes (pronormoblasts, as well as basophilic and polychromatophilic normoblasts) was studied in the circulating blood of round gobies. It was found that the proliferative activity of the erythroid branch of blood-forming tissue in this species increases as the gonads mature, i.e., during transition from the III–IV to the V and VI–II stages of gonad maturity. The content of poorly differentiated cells in the blood grew by 26%, while the number of abnormal erythrocytes (dacryocytes and nuclear invagination) decreased by 3 or 4 times ($R^2 > 0.95$). The content of erythrocyte ghosts, in contrast, increased by 80%. When evaluating the toxicity level of the marine environment, not only the number of abnormal erythrocytes in fish blood but also the activity of the blood-forming tissue should be taken into account, as it substantially varies during the spawning period.

Keywords: abnormal erythrocytes, erythropoiesis, spawning, *Neogobius melanostomus*

DOI: 10.1134/S1063074015020121

INTRODUCTION

Representatives of the Gobiidae family are often used for assessing the impacts of background toxicity on coastal marine waters [1, 5, 21]. They satisfy all the requirements that are imposed on bioindication objects [8, 9]. The magnitude of the toxic effect is usually judged from the state of the circulating blood, which experiences a direct effect of various xenobiotics at the level of respiratory surfaces [2]. These xenobiotics can be a cause of various erythrocyte abnormalities: micronuclei inclusions, cells with nuclear invagination, erythrocyte ghosts, dacryocytes, etc. [2, 14, 15].

Spontaneous hemolysis of mature erythrocytes and cases of nuclear invagination may be intensified under the effects of various toxic agents [6, 15]. An increasing content of dacryocytes is observed, as a rule, during hypoxia and anaemic states [2, 13]. A quantitative evaluation of these and other cell abnormalities provides an opportunity to assess the magnitude of the integral toxic effect.

However, the presence of abnormal erythroid forms in fish blood may also be related to the high proliferative activity of blood-forming tissue (pro- and mesonephros, as well as the spleen) [12]. As is known, erythropoietic processes in fish develop irregularly. The active production of erythrocytes is observed mostly during the post-spawning period, within 3 or 4 months [7, 11]. For the remainder of the time, the blood-forming tissue is not actively functioning. This suggests that an increase in the number of erythrocyte abnormalities in the blood flow of fish does not always reflect the conditions of the marine environment and is rather a consequence of variations in the functional state of the fish. Thus, a regular variation in the number of erythrocytes with nuclear abnormalities within each annual cycle was observed in the gilthead sea bream, *Sparus aurata* [20].

To test this supposition, it is necessary to select batch spawning fish species such as the round goby, *Neogobius melanostomus* Pallas, 1811. In this case, we can obtain individuals at the pre-spawning, spawning, and post-spawning states at the same time, which manifest various rates of proliferative activity of erythropoietic foci.

The goal of the present research is to study the relationship between the contents of abnormal erythrocytes and the number of immature erythroid forms in the circulating blood of the batch-spawning round goby.

MATERIALS AND METHODS

This work was based on adult round gobies at the pre-spawning (III–IV stages of gonad maturity), spawning (V stage), and post-spawning (VI–II stages)

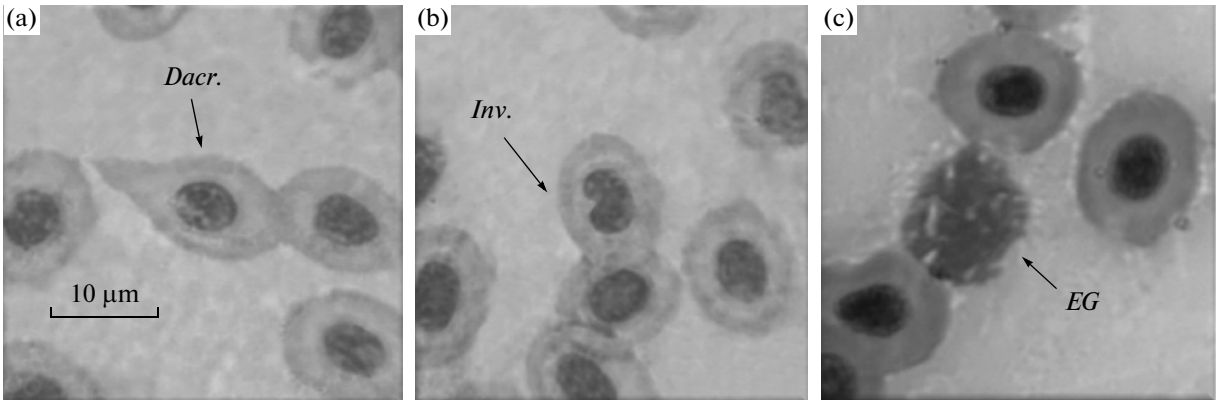

Fig. 1. Abnormal erythroid forms in the blood of the round goby. (a) dacryocytes (*Dacr.*); (b) erythrocytes with nuclear invagination (Inv.); (c) erythrocyte ghosts (*EG*).

states. Gonad maturity stages were differentiated as had been proposed earlier [4]. The spawning state was considered as the one at which the process of gonad maturation ends with the expulsion of sexual products (III–IV → V → VI–II). Fish were caught in the Bay of Sevastopol (Crimea) by using a fixed net and subsequently carried to the laboratory in 50-liter plastic containers with forced aeration. Transport lasted for 30–40 minutes. The material was collected once (June 19, 2013) from one site, i.e., the habitat conditions and, consequently, the probable toxic effect were the same for each of the studied fish groups. The body length of the caught fish was 14–17 cm and the body weight was 30–45 g.

After catching, fish were placed in 50-liter aquariums with a density of 17–25 L water per individual. Natural water flow was provided in the aquariums. The water temperature was kept at 14–16°C; the light–dark cycle was 12 h light/12 h dark. During the experiment, the studied individuals were fed with homogenized fish of low-value species. The daily ration constituted 6–7% of the body weight. Fish were kept under these conditions during 1 week to relieve the stress caused by catching and transport.

Blood was collected through puncturing the caudal artery. As anticoagulant, heparin (Richter, Hungary) was used. Blood samples were collected in 0.5 mL plastic test tubes. Blood smears were prepared and stained according to the combined method by Pappenheim (May-Grünwald + Romanovsky-Giemsa) [3]. The number of immature (pronormoblasts, as well as basophilic and polychromatophilic normoblasts) and abnormal erythroid forms (cells with nuclear invagination, erythrocyte ghosts, and dacryocytes) were counted in all blood smears. The percentage of the cells was found from the counts. The sample size constituted 10000 erythrocytes per smear. A Leica DM1000 light microscope and a Leica DC300 digital camera (Germany) were used in the work.

The statistical processing and graphic visualization of the obtained results were performed by using the Grapher v. 7 standard software package. The results are presented as $\bar{x} \pm S_{\bar{x}}$. The significance of the differences was evaluated using the Student's *t*-test. Pearson's test was used to evaluate the distribution for normality.

RESULTS AND DISCUSSION

Contents of Abnormal Erythroid Forms

The following types of morphological abnormalities of mature erythrocytes were taken into account in examined blood smears: dacryocytes, cells with nuclear invagination, and erythrocyte ghosts.

Dacryocytes are a type of poikilocytosis, i.e., they reflect typical variations in the shape of a mature erythrocyte. They have a drop-like shape and one large projection, called a spicule (Fig. 1a). At the prespawning state (the III–IV stages of gonad maturity), the number of dacryocytes reached 0.5% of the total quantity of erythrocytes (Fig. 2a). As gonads matured (III–IV → VI–II), the number of these cells in the blood of the round goby substantially decreased. In post-spawning individuals (VI–II stages), dacryocytes made up only 0.123 ± 0.052%, which was 4 times lower ($p < 0.05$).

During invagination, the erythrocyte nucleus loses its rounded shape and a typical concavity appears (Fig. 1b). This is believed to be related with breaking the structure of the nuclear membrane [2]. In our case, the number of these cells in the blood of round gobies decreased by 3.2 times ($p < 0.05$) within the spawning period (Fig. 2b). In post-spawning individuals, their proportion constituted 0.424 ± 0.11% of the total quantity of circulating erythrocytes.

Erythrocyte ghosts reflect cases of the lysis of mature erythrocytes in the blood stream. In a blood smear, these look like pinkish spots, which are the

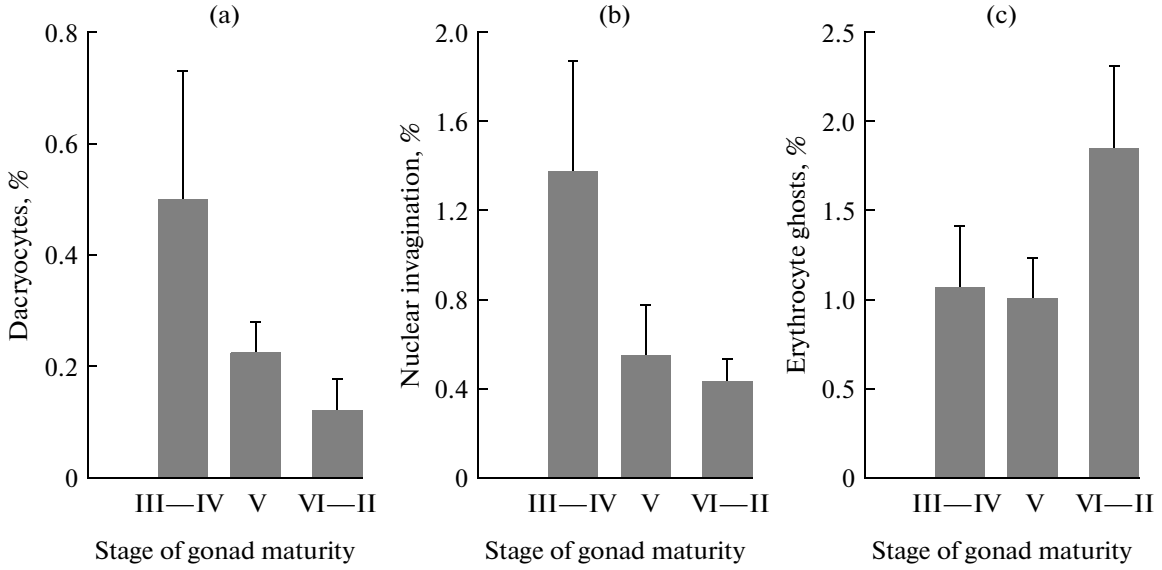

Fig. 2. The content of abnormal erythroid forms in the blood of the round goby depending on the stage of gonad maturity. (a) Dacryocytes; (b) erythrocytes with nuclear invagination; (c) erythrocyte ghosts.

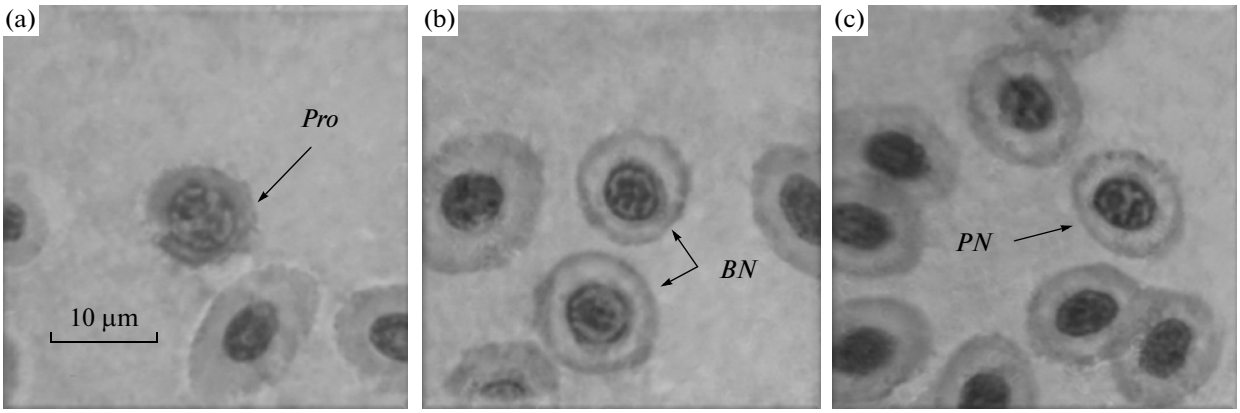

Fig. 3. Immature erythroid forms in the blood of the round goby. (a) Pronormoblast (*Pro*); (b) basophilic normoblast (*BN*); (c) polychromatophilic normoblasts (*PN*).

remains of cell nuclei (Fig. 1c). Normally, this process develops uninterruptedly. Unlike the previous two abnormalities, the content of erythrocyte ghosts in the blood of round goby as gonads matured increased significantly (Fig. 2c). In individuals in the pre-spawning state, they made up about 1% of the number of circulating erythrocytes, whereas after spawning this value grew by 80% ($p < 0.05$).

The Contents of Immature Erythroid Forms in the Blood

The process of maturation and differentiation of erythroid forms in fish includes the following consecutive morphological and functional changes in cells [11, 16]: condensation of nuclear chromatin (heterochromatin) and reduction of the size of the nucleus; a decline in the contents of nucleic acids and accumulation of hemoglobin in the cytoplasm (a change of basophilic staining of the cytoplasm to acidophilic staining); organization of free ribosomes into polyribosome complexes; loss of proliferative activity at the stage of late basophilic normoblast; and the cells acquire an ellipsoid shape. All these can be observed in the following series: pronormoblast → basophilic normoblast → polychromatophilic normoblast (Fig. 3).

As the gonads matured, the proportion of immature erythrocytes in the blood of the round goby increased by 26% ($p < 0.05$) and reached 7.66 ± 0.49% of the total quantity of erythrocytes in the circulating

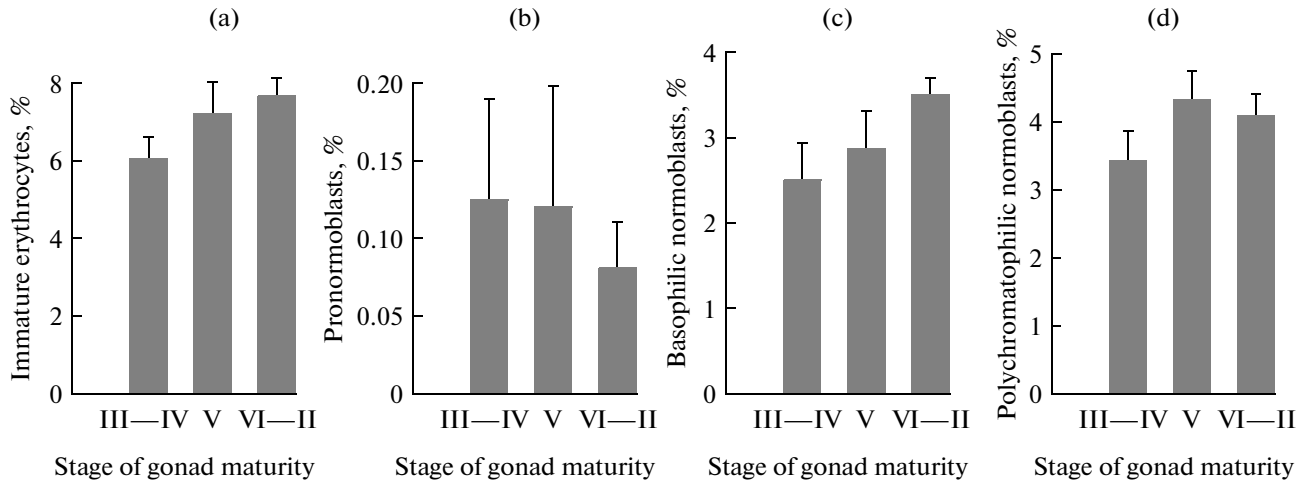

Fig. 4. The content of immature erythroid forms in the blood of the round goby depending on the stage of gonad maturity. (a) The total number of immature erythrocytes; (b) pronormoblasts; (c) basophilic normoblasts; (d) polychromatophilic normoblasts.

blood (Fig. 4a). This took place mainly due to the growth of the level of basophilic and polychromatophilic normoblasts (Figs. 4c and 4d). At the same time, variations in the content of pronormoblasts in fish blood were not significant statistically (Fig. 4b). This type of cell has a pronounced proliferative activity and does not enter the systemic circulation, as they are held by the stroma of the hematopoietic tissue (predominantly pro- and mesonephros) [10]. Finding pronormoblasts in the blood does not enable us to characterize the state of their entire population; the significant variation of the obtained values is related to this as well.

The growth of erythropoietic activity of blood-forming tissue in the round goby during the spawning period may be determined by anemia at the pre-spawning state, which was observed in a number of species of bony fishes under both natural and experimental conditions (injections of hormonal agents) [10, 17, 19]. Anemia is the most powerful factor of the production of erythropoietins. Works on rainbow trout [22] show the possibility of the production of erythropoietins in fish organism. In this species, they were identified in the kidneys, spleen, liver, and in blood plasma using an immunochemical method. The kidneys are probably the site where they form, as the highest concentration of erythropoietins was recorded there. Erythropoietin production in fish was found to correlate positively with the content of sex hormones, particularly testosterone, in the blood [18]. This indicates that spawning and production of erythrocytes by blood-forming tissue are interrelated processes. The state of spawning in fact induces processes of proliferation and differentiation in the erythroid branch of hematopoiesis that is observed during 3–4 months of the post-spawning period [7, 10].

Correlations and Relationships

The contents of abnormal and immature erythroid forms in the blood of the round goby varied during the spawning period, which can be seen from the results of the correlation analysis. The dependence of the number of dacryocytes and cells with nuclear invagination on the level of immature erythroid elements in the blood was inversely proportional at high values of the determination coefficient ($R^2 > 0.95$) (Figs. 5a and 5b). For the content of erythrocyte ghosts, no relationship was found (Fig. 5c).

The functional meaning of the established dependence consists in the fact that, on the one hand, a significant number of relatively young erythrocytes, which still have not been exposed to the toxic effect, enter the blood flow at the moment of active erythropoiesis. The decline in the relative contents of abnormal cells in the blood flow reflects this process. On the other hand, immature erythroid forms are distinguished by their lower osmotic and mechanic durability [10], which apparently determines the increase in the number of erythrocyte ghosts in the circulating blood.

Thus, the active erythrocyte production by blood-forming tissue in the round goby takes place within the spawning period. This becomes evident from the relative growth of poorly differentiated erythroid forms, viz., basophilic and polychromatophilic normoblasts, in the fish blood. This process influences the contents of abnormal cells in the blood: the level of dacryocytes and erythrocytes with nuclear invaginations declines, while the number of erythrocyte ghosts grows. In a number of cases, a clear correlation between these parameters has been established ($R^2 > 0.95$). This means that when counting the number of abnormal erythrocytes in fish blood for the purposes of evaluating the level of toxicity of the marine environment, we

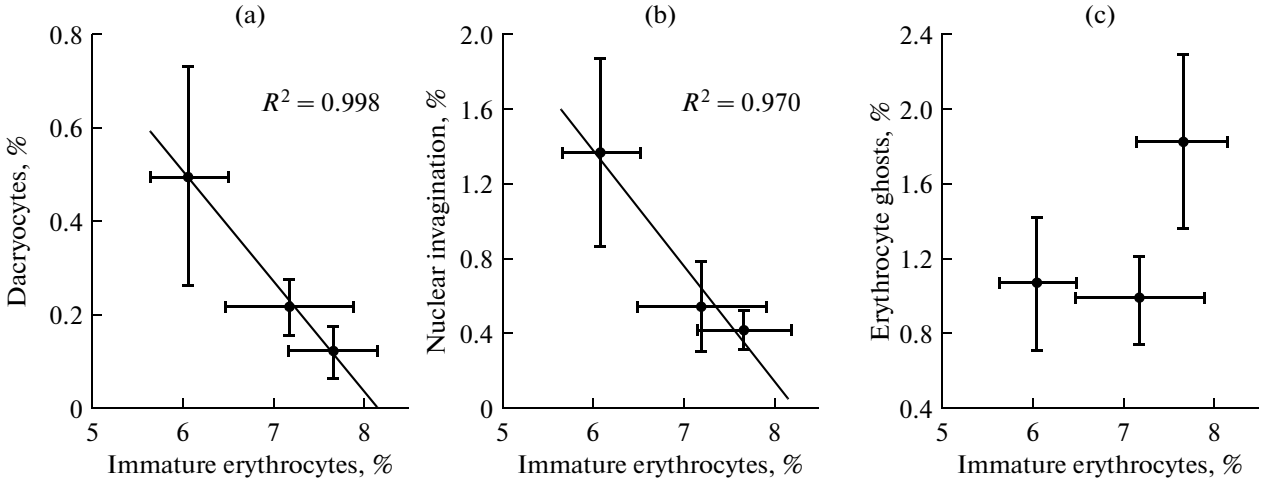

Fig. 5. The dependence of the number of abnormal erythrocytes on the content of immature erythroid forms in the blood of the round goby. (a) Content of immature erythrocytes to content of dacryocytes; (b) content of immature erythrocytes to content of cells with nuclear invagination; (c) content of immature erythrocytes to content of erythrocyte ghosts.

should take the activity of blood-forming tissues into account, which substantially varies within the spawning period.

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Translated by Ye. Shvetsov