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PHYSIOLOGY

Hypoxia-Inducible Factor 1α in the Central Nervous System of the Scallop *Mizuhopecten yessoensis* Jay, 1857 (Bivalvia: Pectinidae) during Anoxia and Elevated Temperatures

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Abstract—An immunocytochemical method was used to study the distribution of hypoxia-inducible factor 1α (HIF-1α) in the central nervous system (CNS) of the scallop *Mizuhopecten yessoensis* (Jay, 1857) (Bivalvia: Pectinidae) under anoxia and elevated temperatures. HIF-1α was not detected in the CNS of control mollusks. After 2 h anoxia, HIF-1α was detected in neurons of the cerebral and visceral ganglia. It was found that a change in HIF-1α immunoreactivity under anoxia had its own dynamics in each of the ganglia; these dynamics depended on the duration of anoxia and the temperature. The presented data indicate the involvement of the transcription factor HIF-1α in providing the compensatory processes that contribute to activation of the neuroprotective mechanisms in the nervous ganglia that control vital functions in *M. yessoensis* under short-term hypoxia.

Keywords: hypoxia-inducible factor-1α (HIF-1α), anoxia, central nervous system, bivalve mollusks

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INTRODUCTION

The ability of marine invertebrates to adapt to changes in oxygen levels is one of their most important adaptations. Studies of the last decade have shown that the regulation of oxygen homeostasis in animals depends greatly on a family of highly conserved transcription factors, where the hypoxia-inducible factor 1 (HIF-1) is of high importance [36–38]. The key factor among these is the oxygen-sensitive factor HIF-1α [15, 22, 33, 50]. It is known that activation of this factor during hypoxia/ischemia causes the expression of genes in mammals that regulate energy metabolism, angiogenesis, erythropoiesis, glycolysis and a number of other processes that provide the adaptation of cells and tissues to low oxygen contents [34, 36, 39, 51]. As well, recent studies have shown that HIF-1α expression plays an important role in maintaining brain homeostasis [36] and neuroprotection processes during a short and moderate effect of ischemia/hypoxia and oxidative stress [25, 41]. A decrease in HIF-1α in the brain may be a factor that causes neuronal death under chronic stress conditions [5].

Analysis of the domain structure of the isoforms of HIF-1α in different animals (nematodes, oysters, and shrimp) and in humans has shown that the main regulatory domains of this protein are homologous in invertebrates and vertebrates, and the homologs of oyster and shrimp HIF-1α resemble the N- and C-terminal oxygen-dependent domains of vertebrates [35]. HIF-1α has been identified in invertebrate animals of different systematic groups by biochemical and molecular methods [11, 13, 17–19, 23, 24, 29, 30, 43]. In addition, a number of studies have been devoted to the study of the role of HIF-1α under hypoxia in organs and tissues of invertebrate animals [13, 17, 23, 30, 40, 43]. However, the expression of HIF-1α in the nervous system has been detected only in hypoxia-tolerant species [19]. There is still no information on the presence, topography, and the number of HIF-1α-expressing cells in the nerve centers that are involved in organization of a stress response in marine hypoxia-sensitive animals. Such species also include the scallop *Mizuhopecten yessoensis* (Jay, 1857), which has a high sensitivity to oxygen deficiency and can be a bioindicator of hypoxia in coastal waters.

The aim of this work was to study the distribution of HIF-1α in the central nervous system (CNS) of the bivalve mollusk *M. yessoensis* under anoxia and at elevated temperatures.

MATERIALS AND METHODS

The study was performed on sexually mature specimens of the scallop *Mizuhopecten yessoensis* (a shell length of 9–10 cm) caught in May 2014 in Peter the

Great Bay (Sea of Japan) at a depth of 2–3 m. To adapt the animals to the experimental conditions, they were kept for several days in an aquarium with aerated seawater at a temperature of 17°C and salinity of 33‰. In the experiment, the mollusks were divided into three groups: the control group (ten scallops) was kept in an aquarium with aerated seawater at a temperature of 17°C under normoxia conditions (the oxygen concentration in the water was 8.5–8.7 mg/L); two experimental groups (ten animals in each) were kept contained in air in closed wet chambers at temperatures of 17 and 25°C, according to the method of Chen et al. [7]. Samples of the nerve tissues were taken after 2-, 4-, 6-, and 12-h anoxia. In our work with hydrobionts, we followed the guidelines of the Council Directive 86/609/EEC of November 24, 1986 and the practical recommendations of Moltschaniwskyj et al. [28].

For immunochemical detection of HIF-1α, the nerve ganglia of mollusks were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4°C. After washing in phosphate buffer, the ganglia were placed for 1 day in a cold 30% sucrose solution in 0.1 M phosphate buffer. HIF-1α was detected in sections (20–25 µm thick) that were cut on a freezing microtome and mounted on object slides. After inhibition of endogenous peroxidase in 1% H_2O_2 solution and on suppression of non-specific antibody binding in 1% normal serum (Vectastain Elite ABC Kit, Vector Labs, United States), the slices were incubated with primary monoclonal antibodies of mouse against HIF-1α (ab16066, Abcam, United States, dilution 1 : 500) for 18 h at 4°C. The sections were then washed in several changes of 0.1M phosphate buffered saline (PBS) (pH 7.2) and incubated for 2 h in a solution of secondary biotinylated antibodies (Vector Labs, United States) at a 1 : 200 dilution. After washing, the sections were incubated with avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector Labs, United States) for 1 h at room temperature in the dark and washed three times in PBS. The reaction products were evaluated with a substrate (VIP Substrate Kit, Vector Labs, United States); the coloration process was controlled under a microscope. The sections were then dehydrated with a standard method and embedded in Canadian balsam. The preparations were examined under an Axiovert 200M microscope (Carl Zeiss, Göttingen, Germany). In the control experiments, we used the negative control method; the primary antibodies were replaced with an equivalent amount of 1% nonimmune serum. In all control experiments, we did not observe an immunopositive reaction.

For quantitative analysis of data, using a series of successive cryostat sections, one section was treated with 1% azure 2 and another was used for immunohistochemical detection of HIF-1α. The number of HIF-1α-positive cells in the ganglia was determined as their proportion of the total number of neurons with the method we described earlier [47]. The mean and the standard error of the mean were calculated from data obtained by processing of at least ten serial sections from each ganglion of control and experimental animals. The data sets for the same ganglia of animals kept at different temperatures were compared using GraphPad Prizm statistical software (version 5.00). The difference between the samples was considered significant at $p \leq 0.05$.

To test the specificity of the immune response, we used the Western blot approach. As a control, the hippocampus of the Wistar rats [9, 48] was used as a control to the samples of the molluscan CNS [9, 48]. To determine the HIF-1α content, we obtained nuclear extracts of rat hippocampus and nerve ganglia taken from five mollusks of each group according to the previously described method [25]. After centrifugation (15000 *g* at 4°C, 20 min), the supernatant (nuclear fraction) was separated, and its total protein concentration was determined by the method of Lowry et al. [26]. Electrophoresis of proteins was carried out in a 12% polyacrylamide gel according to the Laemmli method [20]. The amount of total protein added into the wells for electrophoresis was 30 µg. Markers of 10–175 kDa (GeneDirex, Taiwan) were used as molecular weight markers. After electrophoresis, the proteins were transferred onto a nitrocellulose Whatman membrane (Sigma-Aldrich, United States). Non-specific binding of antibodies was blocked with 4% bovine serum albumin in Tris-HCl buffer (pH 9.0) with 0.1% Tween 20 added overnight at 4°C. The same solution was used for the subsequent dilution of antibodies. The membrane was then incubated with primary monoclonal mouse antibodies against HIF-1α (ab16066, Abcam, United States, dilution 1 : 1000) for 3 h at 4°C. After washing in Tris-HCl buffer, the membranes were incubated for 2 h in a solution of secondary antibodies (Vector Labs, United States). A VIP Substrate Kit (Vector Labs, United States) was used to visualize the peroxidase reaction. The membranes were then washed and scanned.

RESULTS

Identification of the Immunoreactive Product by Western Blotting

Analysis of immunoreactivity did not detect HIF-1α in the total extract of cerebral, visceral, and pedal nerve ganglia of the control individuals of *Mizuhopecten yessoensis* under conditions of normoxia (Fig. 1). Under anoxia of mollusks exposed at 17 and 25°C, in the total extract of nerve ganglia we revealed a polypeptide antigen with a molecular weight of approximately 120 kDa that cross reacts with antibodies to mouse HIF-1α.

Immunohistochemistry

In the central nervous system, which is represented in *M. yessoensis* by paired cerebral and pedal ganglia and an unpaired visceral ganglion [2], no HIF-1α-

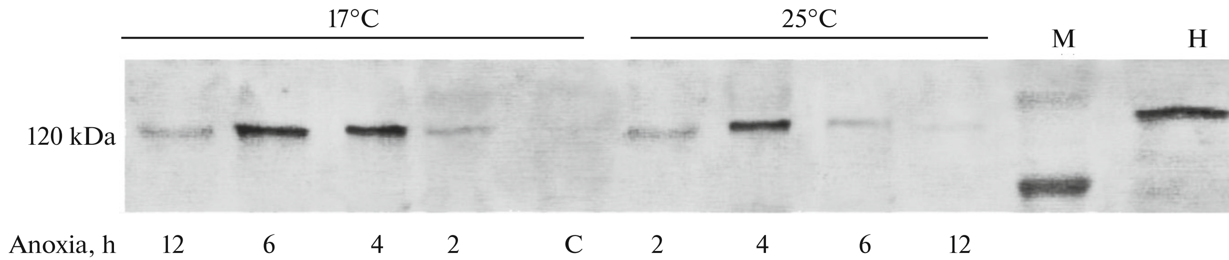

Fig. 1. Western blot analysis of HIF-1α-immunoreactivity in the central nervous system of *Mizuhopecten yessoensis* in anoxia and at temperatures of 17 and 25°C. Legend: M, molecular weight markers, kDa; C, control (normoxia); H, hippocampus of the rat.

immunoreactivity was detected under normoxia (Fig. 2a). Under anoxia, HIF-1α-immunoreactivity was detected in the cerebral and visceral ganglia, where significant local differences were observed in the distribution and contents of HIF-1α-positive cells, which depended on the temperature and duration of the exposure (Figs. 2 and 3). After 2 h of anoxia in both experimental groups of mollusks exposed at 17 and 25°C, HIF-1α-immunoreactivity was detected in the cerebral ganglia, mainly in the neurons of the anterior lobes (Figs. 2d and 2e). During this period, in mollusks exposed at 25°C, HIF-1α-immunoreactivity also occurred in neurons of the visceral ganglion, where mainly the nuclei of large neurons were marked (Fig. 2b). In the pedal ganglia of *M. yessoensis*, HIF-1α-immunoreactivity was not detected after 2 h of the experiment.

After 4 h of anoxia, the number of HIF-1α-positive neurons in the cerebral ganglia of mollusks exposed at 17 and 25°C did not differ statistically (Figs. 2f, 3; $p > 0.05$). In the visceral ganglion, a significant increase in the proportion of the HIF-1α-immunoreactive cells was observed (Figs. 2g–2h, 3; $p < 0.05$). A comparative quantitative analysis showed that in this period in mollusks exposed at a temperature of 25°C, the relative number of HIF-1α-immunoreactive cells was 30% higher than in individuals exposed at a temperature of 17°C, owing to small neurons localized near the neuropil (Figs. 2i–2j, 3). After 6-h anoxia in mollusks exposed at 17°C, the number of HIF-1α-positive cells in the visceral and cerebral ganglia did not change significantly (Figs. 2k, 3; $p > 0.05$). At the same time, in the group of mollusks exposed at 25°C, we recorded the disappearance of immunoreactivity in the cerebral ganglia and a 86% decrease in the proportion of HIF-1α-immunoreactive cells in the visceral ganglion compared to animals after 4-h anoxia (Figs. 2l, 2m, 3; $p < 0.05$). At this stage of the experiment, we also observed opening of the shell valves and the death of individual animals.

After 12 h of anoxia in mollusks exposed at 17°C, the proportion of HIF-1α-immunoreactive cells in the visceral and cerebral ganglia decreased (Figs. 2n and 3).

DISCUSSION

In this study, we observed the absence of HIF-1α in the central nervous system of *Mizuhopecten yessoensis* at normoxia and a local increase in HIF-1α-immunoreactivity under anoxia. The data are consistent with the results of experimental studies that showed the expression of HIF-1α under hypoxic conditions in the peripheral organs of animals of different systematic groups, including bivalve and gastropod mollusks [11, 13, 18, 19, 23, 30, 40, 43]. It was found in our study that HIF-1α-immunoreactivity in all experimental mollusks was detected in the initial period of anoxia primarily in neurons of the cerebral ganglia. The high sensitivity to hypoxia in ganglia of bivalve mollusks has been experimentally proven [16]; data on the involvement of cerebral ganglia in respiratory metabolism are also found in the literature [27, 49]. In the visceral ganglion of *M. yessoensis*, the most significant changes in HIF-1α-immunoreactivity were observed in anoxia; mollusks exposed at a temperature of 25°C exhibited a more rapid and significant increase in HIF-1α than in mollusks exposed at 17°C. It is known that the visceral ganglion in bivalve mollusks is a physiological center that participates in the regulation of respiration, controls motor behavior, and plays an important role in metabolic processes [2]. The expression of the factor HIF-1α in the cerebral and visceral ganglia of the scallop is probably connected with participation of this factor in the adaptation of neurons to hypoxia.

An increase in the content of the HIF-1α in the brain of mammals correlates with neuroprotective reactions and prevents damage or reduces its level under moderate hypoxia/ischemia [25, 41, 44]. A number of studies have shown that an increase in the expression of the HIF-1α in neuronal nuclei indicates activation of the genetic apparatus, proadaptive target-genes and, first of all, genes regulating energy metabolism [51]. It is known that in mammals the HIF-1α participates in adaptation to hypoxia through increased oxygenation of tissues during transition from aerobic to anaerobic energy production, and also as a result of enhanced synthesis of glycolytic enzymes, which are necessary for increasing the yield of enzymatic ATP [12, 42, 45]. These mechanisms in

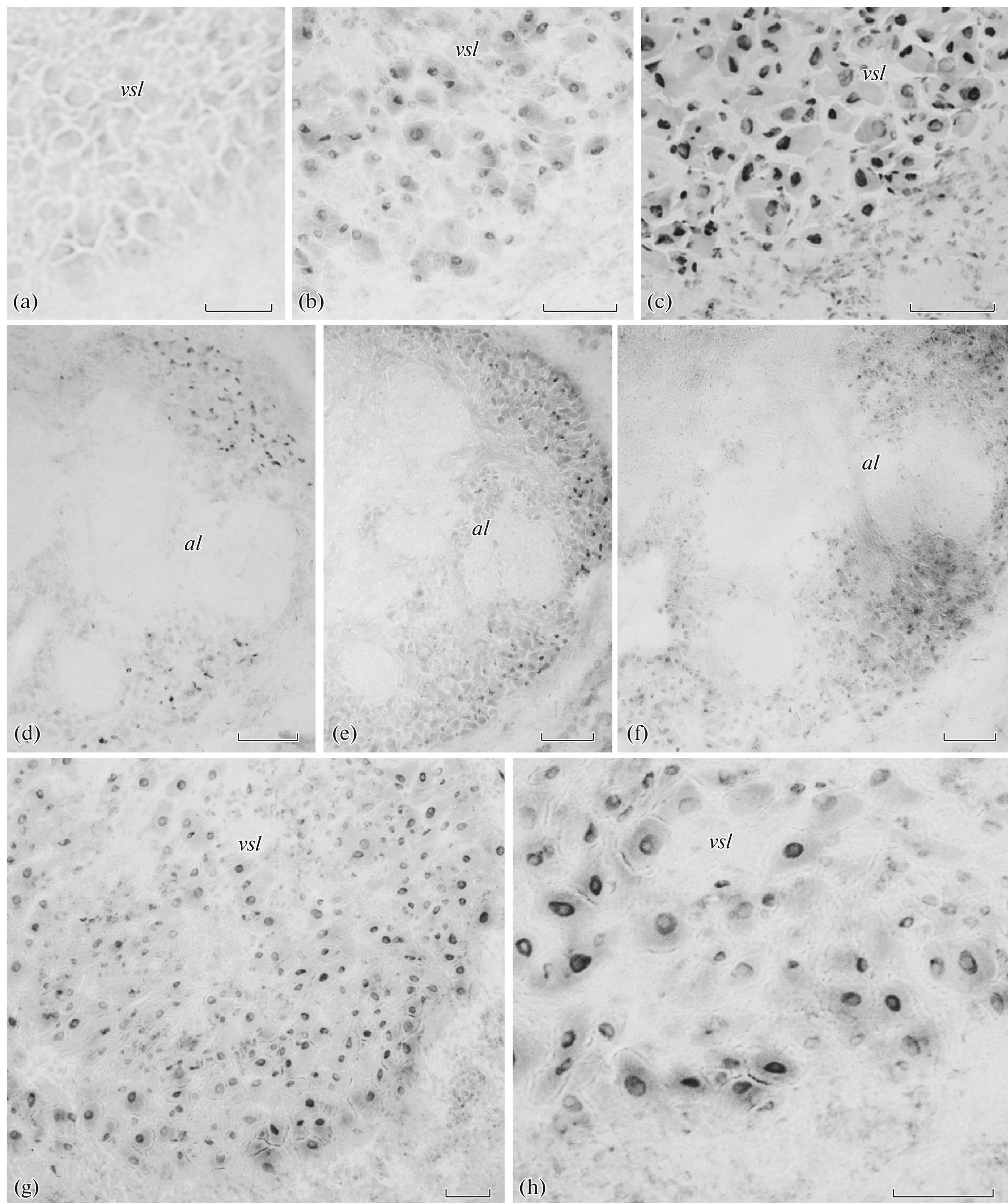

Fig. 2. Distribution of HIF-1α-immunoreactivity in the visceral (a–c, g–n) and cerebral (d–f) ganglia of *Mizuhopecten yessoensis* under anoxia and elevated temperature. (a) Control; (b) 2-h anoxia at 25°C; (c) 2-h anoxia at 17°C, coloration of neurons with azure 2; (d) 2-h anoxia at 25°C; (e) 2-h anoxia at 17°C; (f) 6-h anoxia at 25°C; (g, h) 4-h anoxia at 17°C; (i–j) 4-h anoxia at 25°C; (k) 6-h anoxia at 17°C; (l, m) 6-h anoxia at 25°C; (n) 12-h anoxia at 17°C. Legend: *al*, anterior lobe of the cerebral ganglion; *vcl*, ventrocentral lobe of the visceral ganglion; *n*, neuropil. Scale: 50 μm.

the mammalian brain provide quick and adequate responses to hypoxic stress by stimulation of the respiratory and vasomotor centers, lead to the induction of genes necessary to ensure energy metabolism in cells [10, 51].

In the invertebrate animals, the neuroprotective effect of the HIF-1α has not been yet studied, although there is presently an evidence of HIF-1α expression in the brain of the mantis shrimp *Oratosquilla oratoria* during adaptation to chronic hypoxia

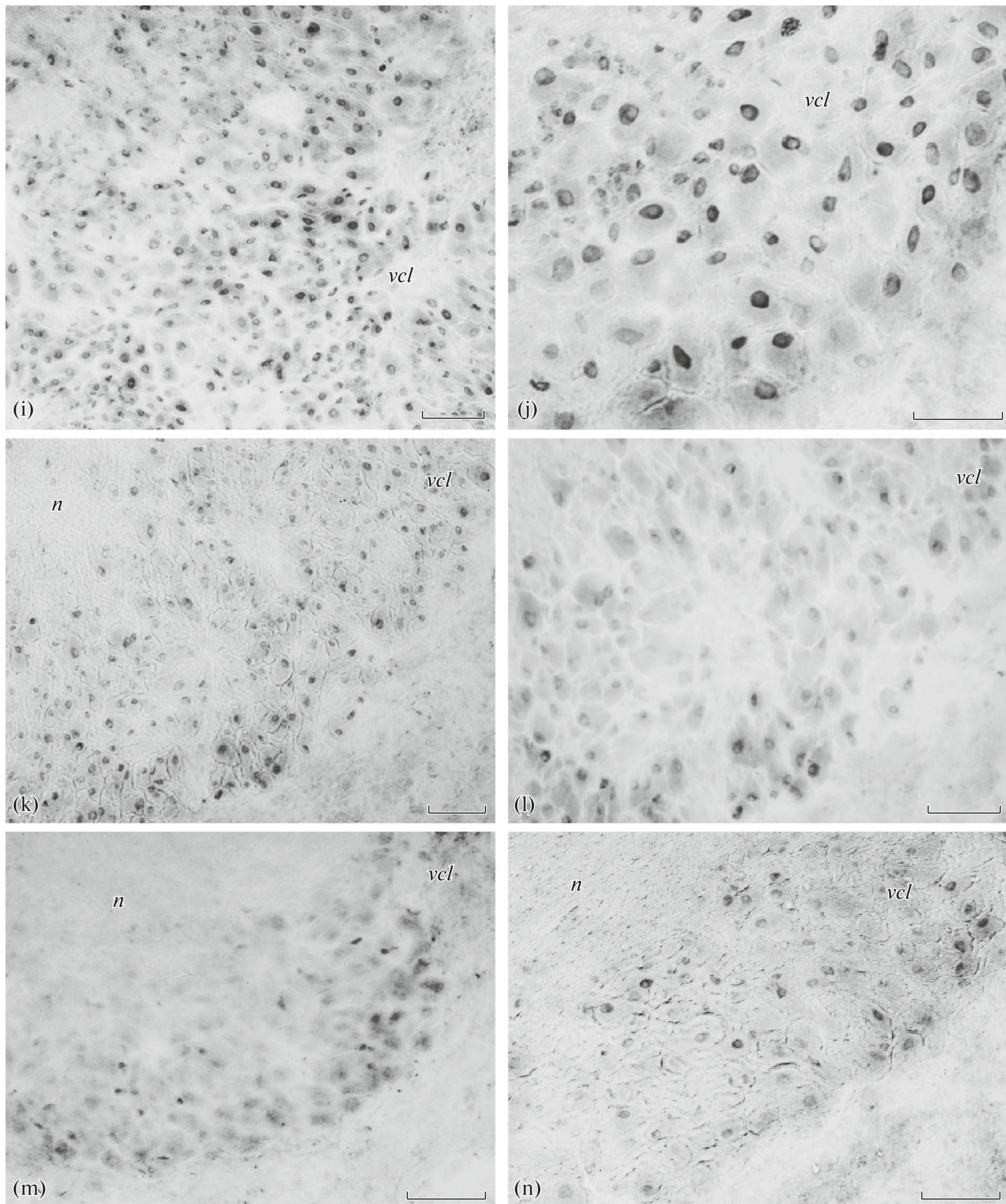

Fig. 2. (Contd.)

caused by anthropogenic pollution of the habitat [19]. In the CNS of the nematode *Caenorhabditis elegans,* Chang and Bargmann [6] identified HIF-1α-immunoreactive neurons, which are involved in behavioral and metabolic reactions under hypoxia. In contrast to mammals, in most invertebrate animals, including mollusks, the survival strategy under hypoxia is directly connected with a decrease in the total ATP consumption [12, 21]. This ability of mollusks to maintain their internal energy resources, switch to anaerobic metabolic pathways, or to reduce the rate of total metabolism through metabolic depression in

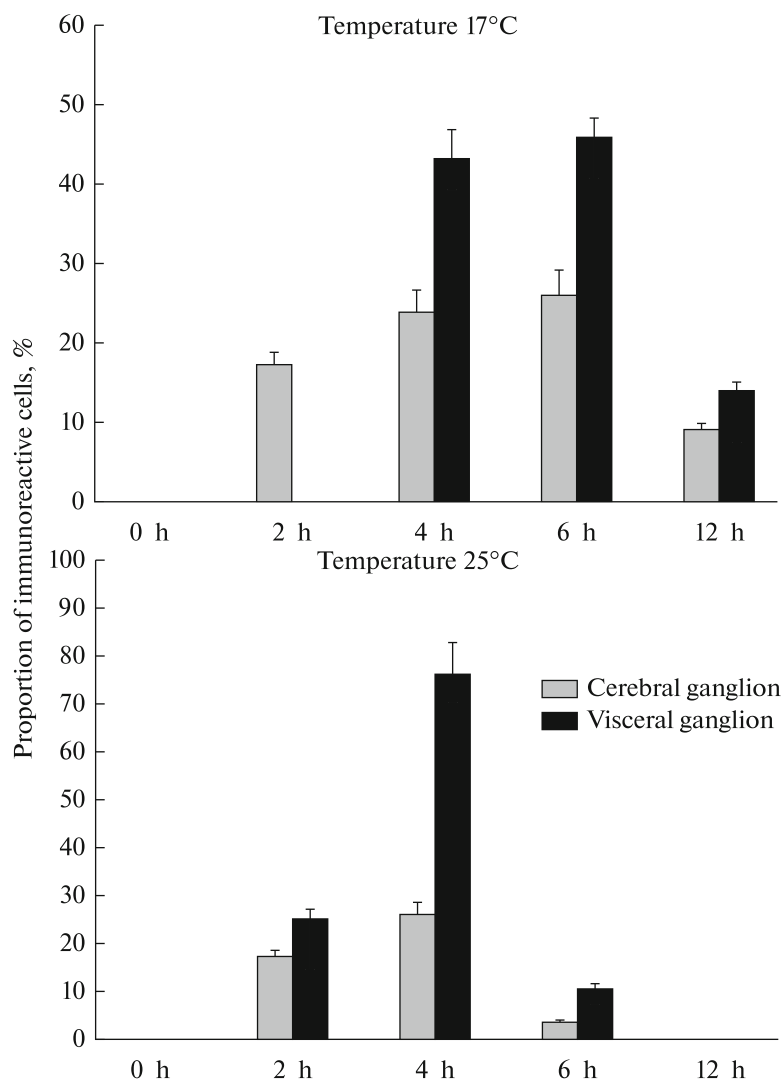

Fig. 3. The proportion of HIF-1α-immunoreactive cells in the CNS of *Mizuhopecten yessoensis* in anoxia and at different temperatures. Vertical lines: the standard error.

response to the effect of low oxygen concentrations in water has been reported in many studies [4, 8, 14, 21]. It was previously found for *Mytilus galloprovincialis* that expression of HIF-1α in the peripheral tissues increased after an 8-h anoxia [11]. However, in the *M. yessoensis* we examined, HIF-1α-immunoreactivity in cerebropleural and visceral ganglia increased after 2 h of anoxia, with each of the ganglia having its own dynamics of changes, which depended on the duration of anoxia and on temperature. These results show that under conditions of acute oxygen deficiency, HIF-1α-immunoreactivity increased in the ganglia, which control the critical functions of the body (respiration, cardioactivity, etc.) that are necessary for survival. This may be connected either with the development of compensatory mechanisms aimed at maintaining aerobic processes, or with switching to anaerobic metabolic pathways. Our data confirm the results of the study of the respiratory metabolism and the activity of oxidative enzymes in the scallop *Pecten maximus* and the mussels of *Mytilus* spp. under the influence of hypoxia and at temperatures of 18 and 25°C [3]. It has been shown that, in contrast to *Mytilus* spp., for which a lack of energy-consuming mechanisms is characteristic [32], in *P. maximus* the intensity of respiration increased after 1 to 4 h of anoxia or a combined action of anoxia and elevated temperature in order to maintain aerobic metabolism [3]. Accordingly, it was found for *P. maximus* that at a decrease in the O_2 level, a stable state was maintained by an increased respiratory rate and cardiac contractions up to the threshold pCO_2 when the compensatory mech-

anisms of the oxygen-transport system were exhausted and anaerobic metabolism was triggered [31]. As well, *P. maximus* has a more significant decrease in aerobic capacity than that in *Mytilus* spp., especially under complex effects of temperature and anoxia [3].

The significant reduction in HIF-1α-immunoreactivity in the ganglia of *M. yessoensis* after 6-h anoxia and the death of mollusks exposed at 25°C seem to be associated with the elevated temperature at which the degree of cellular hypoxia is exacerbated and the level of the HIF-1α falls with an increase in the rate of its degradation, which correlates with a decrease in the function of mitochondria and apoptosis [46]. This assumption is also supported by the data we obtained earlier on the morphological changes in the structure of neurons in *M. yessoensis* under complex effects of temperature and hypoxia [1].

Thus, the increase in HIF-1α-immunoreactivity in the neurons of the visceral and cerebral ganglia of the scallop appears to provide compensatory processes that enhance the adaptive capacity of neurons and promote the activation of neuroprotective mechanisms in the nerve ganglia that control the functions necessary for the survival of the mollusk under hypoxia conditions. Under the influence of an additional stress factor (increased temperature), the efficiency of these mechanisms decreased.

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