

CHANGES OF GLOBINS EXPRESSION IN TONGUE SOLE, *CYNOGLOSSUS SEMILAEVIS* (ACTINOPTERYGII: PLEURONECTIFORMES: CYNOGLOSSIDAE) IN RESPONSE TO SHORT-TERM HYPOXIA

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Background. Fishes live in the aquatic systems which are first affected by low or temporally changing O₂ availability. Under hypoxia, the fish possess varieties of mechanism to ensure the uptake of an adequate amount of O₂. Tongue sole, *Cynoglossus semilaevis* Günther, 1873, lives on the bottom of water where contain low O₂ concentration, indicating that this species possess specific feature against hypoxia. In this study, we examined the mRNA expression of three globins in tongue sole under short-term hypoxia.

Materials and methods. Using bioinformatics analysis, three globin genes including hemoglobin- $\alpha 1$ (Hb- $\alpha 1$), myoglobin (Mb), and cytoglobin (Cygb) were first obtained from tongue sole EST database. Then, their expression levels in different tissues of tongue sole under short-term hypoxia were detected using quantitative real-time PCR method.

Results. Under short-term hypoxia, Hb- $\alpha 1$ significantly increased in all the tissues except intestine and stomach. The Cygb expression level was up-regulated from 5 min to 120 min, whilst changed to normal level at 36 h. Mb was only up-regulated in blood and heart at 120 min. But, as the hypoxia prolonged, there was a mild increase of Mb in blood, heart, spleen, and liver.

Conclusion. The three globins of tongue sole were up-regulated in some tissues, but their role may be different in different stage of hypoxia. Hb and Cygb might play role in the earlier stage, while Mb might play role in the late stage of hypoxia.

Keywords: hemoglobin, cytoglobin, myoglobin, hypoxia, *Cynoglossus semilaevis*

INTRODUCTION

Aquatic hypoxia, a complex ecological phenomenon, can cause major metabolic changes and influence foetal growth and development of all organisms living in water (Kajimura et al. 2004). With the increased industrialization and urbanization of coastal zones in recent years, hypoxia has grown strikingly in aquatic systems and has received much attention in biomedical research (Gray et al. 2002, Wawrowski et al. 2011).

Fishes are the main aquatic animals that are first affected by hypoxia, and so fishes have become a prime model to investigate hypoxia tolerance strategies at the organism level (Roesner et al. 2008). Under hypoxia, a variety of physiological, anatomical and behavioural mechanisms were activated in fish that enables fish to survive in this condition. One of the main mechanisms relies on the globins to enhance the O₂ transport capacitance,

facilitate intracellular O₂ diffusion, or enable O₂ storage for hypoxia. To date, many types of globins have been identified in vertebrates. Hemoglobin (Hb) is a heterotetramer that consists of two α and two β chains. Hb is located in the erythrocytes and serves for the transport of O₂. Myoglobin (Mb) is a monomeric protein and mainly included in the myocytes of the heart and skeletal muscles to store oxygen and to facilitate intracellular oxygen diffusion (Wittenberg and Wittenberg 2003). Neuroglobin (Ngb) is mainly resided in the central and peripheral nervous system (Burmester et al. 2000) and may supply O₂ to metabolically active neurons. Cytoglobin (Cygb) is expressed in the fibroblast cells and distinct neurons, and may be involved in reactive oxygen species detoxications or in the function of supplying oxygen to particular enzymatic reactions (Burmester et al. 2002, Schmidt et al. 2004). Those four globins (Hb, Mb, Ngb, and Cygb) have been

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characterized in most vertebrates including mammals, fishes, and amphibians. Other globins seem to be restricted to some vertebrate taxa. Globin E (GbE) is an eye-specific globin and only found in birds (Blank et al. 2011). Globin X (GbX) is only found in amphibians and fishes. GbX is bound to the cell membrane and possess a protective function. Globin Y (GbY) was only presents in *Xenopus laevis* and has a broad expression pattern in *Xenopus* tissues (Fuchs et al. 2006). The exact function of GbY remains unknown.

Due to its pleasant taste and high economic value, tongue sole, *Cynoglossus semilaevis*, is becoming an increasing important marine fish species in China. Meanwhile, the genomic sequence of tongue sole has been completed recently, and this fish will be used as a model for sex determination mechanism and other biomedical research (Shao et al. 2010). The tongue sole lives on the bottom of water where contain low O₂ concentration, indicating that this species possess its specific feature against hypoxia. However, response of tongue sole under hypoxia remains unclear. In the present study, the information of the globins including Hb- α 1, Mb and Cygb was first obtained by analyzing the tongue sole EST database. Then, their expression patterns in response to short-term hypoxia were examined.

MATERIALS AND METHODS

Experimental fish. Adult tongue sole, *Cynoglossus semilaevis*, were purchased from a local hatchery and kept for 2 weeks in a large tank with a seawater recirculation system in our laboratory. Prior to use, the fish were maintained on a 14-h light / 10-h dark cycle at 21°C and fed twice daily with a commercial feed. The YSI multiprobe (YSI 556 Multiprobe system) was used to monitor dissolved oxygen (DO), pH, and temperature. The water conditions were controlled at the following level: DO = 6.2 mg · L⁻¹, pH = 7.0, total ammonia < 0.2 mg · L⁻¹, and nitrite < 0.02 mg · L⁻¹. The Institutional Review Board of the Yancheng Institute of technology, China, approved this study as consistent with the country's regulations on experiments on animals.

Preliminary study of hypoxia in tongue sole. After acclimation, thirty fish were randomly assigned into three 40 L tanks (10 fish per tank) at 21°C. The air stones in each tank were removed and nitrogen gas were bubbled

into the tank to obtain the 0.2 mg · L⁻¹, 0.8 mg · L⁻¹, and 1.2 mg · L⁻¹ DO level, respectively. Then, the vitality of fish in each tank was observed (as expressed by their general motor activity).

Database and sequence analysis. Using the globins from human as bait, the EST database of tongue sole was searched using tBLASTn software. The obtained sequence was translated into amino acids using software at ExPASy molecular biology Server (<http://www.expasy.org>), and further determined using BLASTp software in NCBI database (<http://www.ncbi.nlm.nih.gov>).

Hypoxia treatment. Eighty fish (23–29 cm long and weighing 60–80 g) were randomly transferred into each of seven 40-L tanks with one control group (20 fish) and six experimental groups (10 fish per group) and reared at 21°C. The control group was maintained under normoxic conditions (DO 6.2 mg · L⁻¹), and the experimental groups were reared under hypoxic condition (0.8 mg · L⁻¹) for 5, 30, 60, 90, 120 min, and 36 h, respectively. After the exposure period, three fishes from each group were sampled and tissue samples were subsequently stored at –80°C until used.

RNA extraction, reverse transcription and cDNA cloning. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. To avoid contamination with genomic DNA, the RNA samples was digested with RNase-free DNase I (Takara) incubation for 30 min at 37°C. First-strand cDNA was synthesized using a SuperScriptTM Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. The cDNA of interest genes were amplified by PCR using the ExTaq enzyme (TaKaRa, Japan) and primers listed in Table 1. The cDNA fragments were cloned into pMD18-T vector and sequenced by a commercial sequencing service.

Quantitative real-time PCR. Total RNA was isolated from the each sample using Trizol Reagent (Invitrogen). The genomic DNA in the RNA samples was digested with RNase-free DNase I (Takara) incubation for 30 min at 37°C. 2 μ g RNA was transcribed into cDNA using PrimeScript[®] RT reagent kit (TaKaRa, Japan). All cDNA samples were stored at –20°C until being used.

Tongue sole β -actin (GenBank accession No. GH234955), Hb- α 1 (GenBank accession No. HQ909440), Mb (GenBank accession No. GH230970) and Cygb

Primers used in the study on globins in *Cynoglossus semilaevis*

Table 1

Primer designation	Sequence	Product size [bp]	Annealing temperature [°C]
Hb- α 1	Forward: 5'-AACCAAGACCTACTCCACCCA-3'	147	59
	Reverse: 5'-CTTCTCGCTGAGGTCCAACA-3'		
Cygb	Forward: 5'-GATGCAGGAAGAGGTGGCTCAC-3'	263	64
	Reverse: 5'-CCAGCGTGTGAGGGAGTTCAT-3'		
Mb	Forward: 5'-ATCCAGCCTCTAGCCCAATCAC-3'	203	64
	Reverse: 5'-CCCTGGAAGCCTAGCTCCTTGT-3'		
β -actin	Forward: 5'-CAGCCATACTGTGCCCATCT-3'	165	59
	Reverse: 5'-TCCTTGATGTCACGCACGAT-3'		

(GenBank accession No. GH231385) fragments were obtained using RT-PCR, purified, cloned into pMD18-T vector and confirmed by sequencing. The plasmid DNA was extracted using QIAGEN plasmid mini kit and the concentrations were measured by spectrophotometer at OD_{260} . The corresponding copy number was calculated as $1 \mu\text{g}$ of 1000-bp DNA = 9.1×10^{11} molecules (Overbergh et al. 2003). Serial ten-fold dilutions of the plasmid, e.g., ranging from 10^8 to 10^2 input cDNA copies, were used as a standard curve in PCR run. Real-time PCR experiments were performed on an ABI 7300 Real-time PCR system (Applied Biosystems). Amplifications were carried out at a final volume of $20 \mu\text{L}$, containing $1 \mu\text{L}$ cDNA template, $10 \mu\text{L}$ SYBR premix ExTaq, $0.4 \mu\text{L}$ $50 \times$ Rox reference dye, $1 \mu\text{L}$ of each primer, and $6.6 \mu\text{L}$ ddH₂O. The PCR amplification were performed in triplicate wells and carried out using two-step method: 30 s at 95°C , followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. Specificity of amplification was analyzed using dissociation curves with temperature ranging from 60°C to 95°C . The molecular number of a particular gene transcript was calculated based on the standard curve and normalized to the β -actin level (Qi et al. 2010, 2011). The results were first performed in the ABI Prism 7300 SDS program and final analyzed with the Microsoft Excel. The significance of the data was evaluated using Student's *t*-test. All the primers used for this analysis were listed in Table 1.

RESULTS

Hypoxia in tongue sole. To further determine the hypoxic enduring ability of tongue sole, we first monitored the survival status of tongue sole under hypoxia. We applied three different hypoxia regimes, with $\text{DO} = 0.2, 0.8,$ and $1.2 \text{ mg} \cdot \text{L}^{-1}$ for various periods of time. The fish under DO of $0.8 \text{ mg} \cdot \text{L}^{-1}$ and $1.2 \text{ mg} \cdot \text{L}^{-1}$ survived for up

to 48 h without detectable defects. However, the DO of 0.2 was lethal and the fishes died quickly in less than 15 min. So, the DO of $0.8 \text{ mg} \cdot \text{L}^{-1}$ was applied for short-term hypoxia exposure.

Changes of globins mRNA under hypoxia. The mRNA expression level of globins under $\text{DO} = 0.8 \text{ mg} \cdot \text{L}^{-1}$ in different tissues of tongue sole were determined using real-time PCR method. Here, we observed that after hypoxia stressed for 30 min to 36 h, the expression of Hb- $\alpha 1$ was significantly increased in heart, liver, spleen, kidney, blood, and gills ($P < 0.05$), whilst in intestine only at 30 min were up-regulated ($P < 0.05$) and back to normal level from 60 to 36 h ($P > 0.05$). There was no significantly change of Hb- $\alpha 1$ was observed in stomach from 5 min to 36 h ($P > 0.05$) (Fig. 1). The Cygb mRNA expression level was also up-regulated in heart, liver, spleen, kidney, blood, and gills from 30 min to 120 min hypoxia stressed, and in stomach and intestine only increased at 5 min hypoxia stressed ($P < 0.05$). But, Cygb changed to normal level at 36 h when compared to the normoxic control group ($P > 0.05$) (Fig. 2). The Mb remained unchanged, except increasing in blood and heart at 120 min hypoxia stressed ($P < 0.05$). As the hypoxia prolonged to 36 h, there was a mild increase of Mb in blood, heart spleen and liver ($P < 0.05$) (Fig. 3).

DISCUSSION

In the presently reported study, a marine fish, tongue sole, *Cynoglossus semilaevis*, which lives on the bottom of water, was selected to study its response to hypoxia. Because of its specific habitat, tongue sole need specific mechanism to resist the low O_2 in water. Our results showed that this species could survive at DO of $0.8 \text{ mg} \cdot \text{L}^{-1}$ for up to 36 h, which was longer than that of red seabream, *Pagrus major* (Temminck et Schlegel, 1843), and yellow

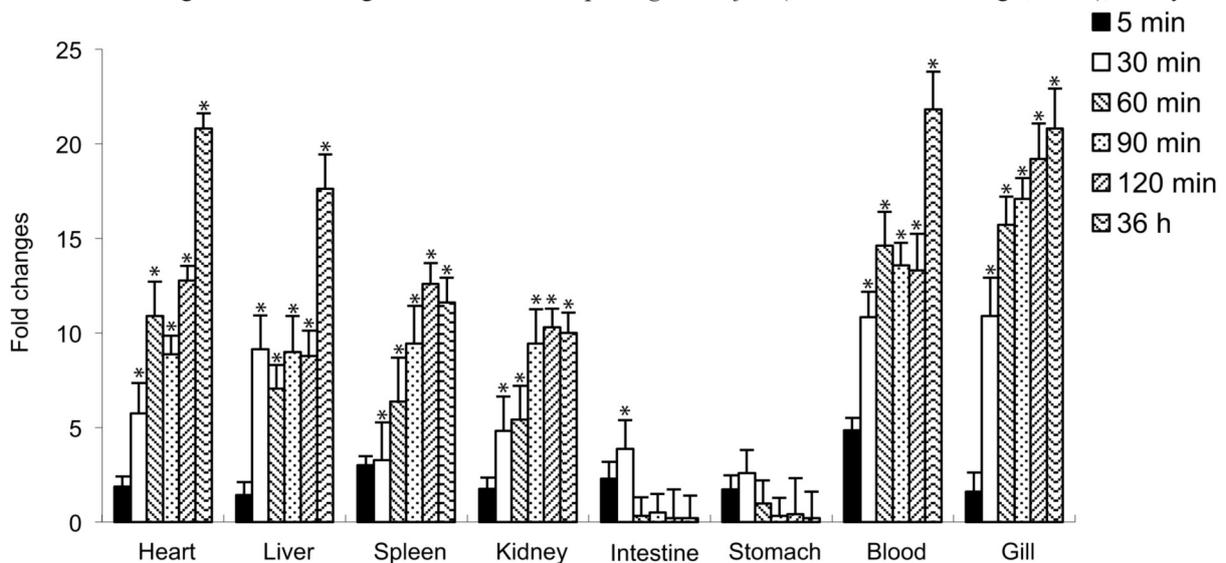


Fig. 1. Relative expression levels of Hb- $\alpha 1$ under hypoxia from 5 min to 36 h in different tissues of *Cynoglossus semilaevis*; The gene expression level was normalized against the housekeeping gene β -actin and the fold change of expression obtained by comparing the normalized expression of the hypoxic group with that of the control group; Asterisks indicate that the copy number is significantly higher than that of the control group ($*P < 0.05$) and data were expressed as the mean \pm standard error of three individuals ($n = 3$)

grouper, *Epinephelus awoara* (Temminck et Schlegel, 1842), indicating tongue sole has better ability to adapt the low oxygen.

Fish had developed many strategies to adapt the environmental changes and enable them to survive under hypoxia (Childress and Seibel 1998, Richards 2011). Many genes encoding enzymes of the glycolytic pathway and fermentation were strongly expressed (Ton et al. 2003, Van den Meer et al. 2005), while the genes encoding the oxygen-dependent energy production and protein translation was inhibited. Meanwhile, some respiratory proteins e.g., globins in fish were also affected by hypoxia (Nikinmaa and Rees 2005, Roesner et al. 2008).

In fish, five types of globins have been identified including Hb, Mb, Ngb, Cygb, and GbX. By using bioin-

formatics method, we obtained three globins, Hb- $\alpha 1$, Mb, and Cygb from the EST database of tongue sole. Those three genes shared higher identities with their counterparts in fish (data not shown), confirming those genes were the Hb- $\alpha 1$, Mb, and Cygb homologous. Unfortunately, we did not obtain two other globins, reason for might be the incompleteness of tongue sole genome sequence.

Although hemoglobins were conserved during evolution and shared high homologies in different fish (Borza et al. 2009, Quinn et al. 2010), there was no common expression pattern for piscine Hbs under hypoxia. Some studies found an up-regulated of Hbs under hypoxia (Timmerman and Chapman 2004, Wawrowski et al. 2011), while others found un-change in Hb levels (Roesner et al. 2008). In this

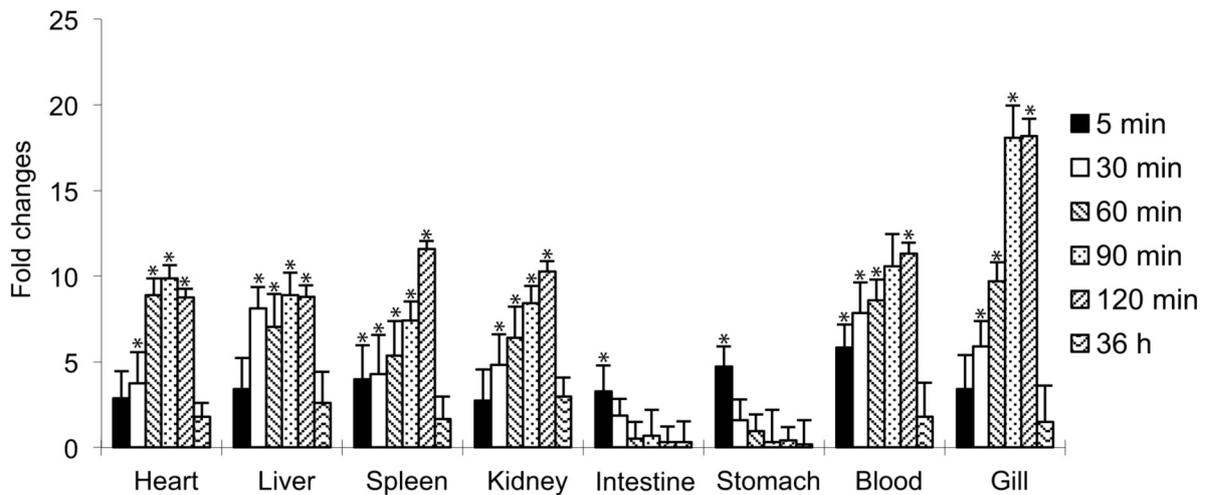


Fig. 2. Relative expression levels of Cygb under hypoxia from 5 min to 36 h in different tissues of *Cynoglossus semi-laievis*; The gene expression level was normalized against the housekeeping gene β -actin and the fold change of expression obtained by comparing the normalized expression of the hypoxic group with that of the control group; Asterisks indicate that the copy number is significantly higher than that of the control group ($*P < 0.05$) and data were expressed as the mean \pm standard errors of three individuals ($n = 3$)



Fig. 3. Relative expression levels of Mb under hypoxia from 5 min to 36 h in different tissues of *Cynoglossus semi-laievis*; The gene expression level was normalized against the housekeeping gene β -actin and the fold change of expression obtained by comparing the normalized expression of the hypoxic group with that of the control group; Asterisks indicate that the copy number is significantly higher than that of the control group ($*P < 0.05$) and data were expressed as the mean (\pm standard errors) of three individuals ($n = 3$)

study, we found that in tongue sole Hb- α 1 mRNA levels was increased under hypoxia. Unlike human, teleost do not have bone marrow. The haematopoietic tissue of fish is mainly found in liver, kidney, spleen and other tissues (Willet et al. 1999). Under hypoxic stressed, the fish could activate the haematopoiesis process to rise of Hb concentration to increase the O₂-transport capacity of the blood and enhance O₂ supply to the inner organs (Wawrowski et al. 2011), and this might be the exact reason for the increasing of Hb.

The expression of Cygb was related to proliferation and reactive oxygen species detoxification or O₂ supply to particular enzymatic reactions (Schmidt et al. 2004). Data from piscine Cygb was little and it was difficult to rule its expression pattern. Here, we observed that Cygb was increased in some tissues after hypoxia stressed for 5 to 120 min and back to normal level after 36 h, indicating that Cygb might play a role in the earlier stage of hypoxia.

Mb was first identified as a muscle-specific tissue-globin that supports intracellular O₂ supply (Wittenberg and Wittenberg 2003). However, recent studies showed that it also expressed in non-muscle tissues (Fraser et al. 2006, Wawrowski et al. 2011). Our study observed that the tongue sole Mb was expressed in liver, spleen, gill, blood, and heart, giving light that Mb might play a role in other tissues except in cardiac tissue. Although the data on the level of mammals Mb under hypoxia remains un-constant (Levine and Stray-Gundersen 2001), information from teleost fish including common carp, *Cyprinus carpio* L.; goldfish, *Carassius auratus* (L.); (see Roesner et al. 2008); zebrafish, *Danio rerio* (Hamilton, 1822); (see Roesner et al. 2006); and Japanese medaka, *Oryzias latipes* (Temminck et Schlegel, 1846) (see Wawrowski et al. 2011) was constant that Mb was increased under hypoxia. In this work, we observed that the tongue sole Mb level was increased in some tissues until 120 min or 36 h after hypoxia, indicating Mb might play a role in the late stage of hypoxia. In contrast to some inner tissues, muscle cells can survive via anaerobic fermentation under hypoxia; so, the delayed increase in Mb concentration is sufficient to ensure the supply of O₂ to the muscle cells.

In conclusion, three globins were first identified from the marine fish, tongue sole, and their expression pattern under short-term hypoxia were examined by using real-time PCR method. The results showed the three globins were up-regulated in some tissue, but their role might be different in different stage of hypoxia. Hb- α 1 and Cygb might play role in the earlier stage, while Mb might play role in the late stage of hypoxia.

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REFERENCES

- Blank M., Kiger L., Thielebein A., Gerlach F., Hankeln T., Marden M.C., Burmester T.** 2011. Oxygen supply from the bird's eye perspective: globin E is a respiratory protein in the chicken retina. *Journal of Biology Chemistry* **286** (30): 26507–26515. DOI: 10.1074/jbc.M111.224634
- Borza T., Stone C., Gamperl A.K., Bowman S.** 2009. Atlantic cod (*Gadus morhua*) hemoglobin genes: multiplicity and polymorphism. *BMC Genetics* **10**: 51–65. DOI: 10.1186/1471-2156-10-51
- Burmester T., Weich B., Reinhardt S., Hankeln T.** 2000. A vertebrate globin expressed in the brain. *Nature* **404**: 520–523. DOI: 10.1038/35035093
- Burmester T., Ebner B., Weich B., Hankeln T.** 2002. Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. *Molecular Biology Evolution* **19**: 416–421.
- Childress J.J., Seibel B.A.** 1998. Life at stable low oxygen levels: adaptations of animals to oceanic oxygen minimum layers. *Journal of Experimental Biology* **201**: 1223–1232.
- Fraser J., Mello L.V., Ward D., Rees H.H., Williams D.R., Fang Y., Brass A., Gracey A.Y., Cossins A.R.** 2006. Hypoxia-inducible myoglobin expression in nonmuscle tissues. *Proceeding of National Academy of Science USA* **103**: 2977–2981. DOI: 10.1073/pnas.0508270103
- Fuchs C., Burmester T., Hankeln T.** 2006. The amphibian globin gene repertoire as revealed by the *Xenopus* genome. *Biochemical and Biophysical Research Communications* **337** (3–4): 216–223. DOI: 10.1159/000089884
- Gray J.S., Wu R.S., Ying Y.O.** 2002. Effects of hypoxia and organic enrichment on the coastal marine environment. *Marine Ecology Progress Series* **238**: 249–279. DOI: 10.3354/meps238249
- Kajimura S., Aida K., Duan C.** 2004. Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. *Proceeding of National Academy of Science USA* **102** (4): 1240–1245. DOI: 10.1073/pnas.0407443102
- Levine B.D., Stray-Gundersen J.** 2001. The effects of altitude training are mediated primarily by acclimatization, rather than by hypoxia exercise. *Advances in Experimental Medicine Biology* **502**: 75–88.
- Nikinmaa M., Rees B.B.** 2005. Oxygen-dependent gene expression in fishes. *American of Journal Physiology Regulatory Integrative and Comparative Physiology* **288**: R1079–R1090. DOI: 10.1152/ajpregu.00626.2004
- Overbergh L., Giulietti A., Valckx D., Decallonne R., Bouillon R., Mathieu C.** 2003. The use of realtime reverse transcriptase PCR for the quantification of cytokine gene expression. *Journal of Biomolecular Techniques* **14**: 33–43
- Qi Z.T., Nie P.** 2008. Comparative study and expression analysis of the interferon gamma gene locus cytokines in *Xenopus tropicalis*. *Immunogenetics* **60** (11): 699–710. DOI: 10.1007/s00251-008-0326-y
- Qi Z.T., Zhang Q.H., Wang Z.S., Wang A.M., Huang B., Chang M.X., Nie P.** 2011. Cloning and expression analysis of a long type peptidoglycan recognition protein (PGRP-L) from *Xenopus tropicalis*. *Zoological Research* **32** (4): 371–378. DOI: 10.3724/SP.J.1141.2011.04371

- Quinn N.L., Boroevich K.A., Lubieniecki K.P., Chow W., Davidson E.A., Phillips R.B., Koop B.F., Davidson W.S.** 2010. Genomic organization and evolution of the Atlantic salmon hemoglobin repertoire. *BMC Genomics* **11**: 539. DOI: 10.1186/1471-2164-11-539
- Richards J.G.** 2011. Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. *Journal of Experimental Biology* **214**: 191–199. DOI: 10.1242/jeb.047951
- Roesner A., Hankeln T., Burmester T.** 2006. Hypoxia induces a complex response of globin expression in zebrafish (*Danio rerio*). *Journal of Experimental Biology* **209**: 2129–2137. DOI: 10.1242/jeb.02243
- Roesner A., Mitz S.A., Hankeln T., Bumester T.** 2008. Globin and hypoxia adaptation in the goldfish, *Carassius auratus*. *FEBS Journal* **275** (14): 3633–3643. DOI: 10.1111/j.1742-4658.2008.06508.x
- Schmidt M., Gerlach F., Avivi A., Laufs T., Wystub S., Simpson J.C., Nevo E., Saaler-Reinhardt S., Reuss S., Hankeln T., Burmester T.** 2004. Cytoglobin is a respiratory protein in connective tissue and neurons, which is up-regulated by hypoxia. *Journal of Biology Chemistry* **279**: 8063–8069. DOI: 10.1074/jbc.M310540200
- Shao C.W., Chen S.L., Scheuring C.F., Xu J.Y., Sha Z.X., Dong X.L., Zhang H.B.** 2010. Construction of two BAC libraries from half-smooth tongue sole *Cynoglossus semilaevis* and identification of clones containing candidate sex-determination genes. *Marine Biotechnology* **12** (5): 558–568. DOI: 10.1007/s10126-009-9242-x
- Timmerman C.M., Chapman L.J.** 2004. Behavioral and physiological compensation for chronic hypoxia in the sailfin molly (*Poecilia latipinna*). *Physiological and Biochemical Zoology* **77**: 601–610.
- Ton C., Stamatou D., Liew C.C.** 2003. Gene expression profile of zebrafish exposed to hypoxia during development. *Physiological Genomics* **13**: 97–106. DOI: 10.1152/physiolgenomics.00128.2002
- Van den Meer D.L., van den Thillart G.E., Witte F., de Bakker M.A., Besser J., Richardson M.K., Spink H.P., Leito J.T., Bagowski C.P.** 2005. Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. *American Journal of Physiology Regulatory Integrative and Comparative Physiology* **289**: R1512–R1519. DOI: 10.1152/ajpregu.00089.2005
- Wawrowski A., Gerlach F., Hankeln T., Burmester T.** 2011. Changes of globin expression in the Japanese medaka (*Oryzias latipes*) in response to acute and chronic hypoxia. *Journal of Comparative Physiology B* **181**: 199–208. DOI: 10.1007/s00360-010-0518-2
- Wittenberg J.B., Wittenberg B.A.** 2003. Myoglobin function reassessed. *Journal of Experimental Biology* **206**: 2011–2020. DOI: 10.1242/jeb.00243

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