

Superoxide dismutase and oxygen transport mechanism in endemic fish *Delminichthys ghetaldii* (Steindachner, 1882) under mild hypoxia

Jasna Frišćić¹, Radoslav Dekić², Aleksandar Ivanc³ & Biljana Kukavica^{2*}

¹Universitätsklinikum Erlangen: Lübeck, Schleswig-Holstein, Germany

²Faculty of Natural Science and Mathematics, University of Banja Luka, Mladena Stojanovica 2, Banja Luka, Bosnia and Herzegovina

³Department of Biomedical Sciences, State University of Novi Pazar, Vuka Karadzica bb, Novi Pazar, Serbia

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Hypoxia, a condition when dissolved oxygen (DO) drops down below 2 mg/L¹, is one of the most common stressors in aquatic environment. Aquatic animals often overcome this condition with a combination of physiological, behavioural, biochemical and molecular responses. In the present study, we examined the effect of moderate hypoxia on superoxide dismutase (SOD) activity and oxygen transport mechanism in *Delminichthys ghetaldii* erythrocytes. Popovo minnow (*Delminichthys ghetaldii*) is an endemic cyprinid fish which inhabits springs and streams in fields Ljubomir, Dabar and Fatnica in B&H, and Bacina lakes in Croatia. During autumn and spring floods, Popovo minnows emerge from the underground waters to surface estavelle. Withdrawal of the water to the underground basins brings them back to their underground habitat. Fishes were collected in the flood plain of Fatnica field (43°00'53"N; 18°19'24"E). Following erythrocyte lineage parameters were measured: number of red blood cells (RBC), hemoglobin concentration, Packed cell volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin Concentration (MCHC) and MCH (Mean Corpuscular Hemoglobin). Also, the characterization of the SOD from RBC is presented for the first time. Native PAGE resolved the presence of three CuZn SOD isoforms, while isoelectrofocusing revealed the presence of two anionic SOD isoforms with pI 3.4 and 3.5. Exposure to conditions of mild hypoxia induced changes in fish SOD activity, hemoglobin concentration and MCH. Results indicate *Delminichthys ghetaldii* responds to hypoxia by enhancement of the oxygen carrying capacity and triggering SOD activity.

Keywords: Antioxidant enzymes, Karst, Popovo minnow, Reactive oxygen species, Water pollution

Hypoxia is one of the most common stressors related to aquatic environments and it occurs when the concentration of dissolved oxygen (DO) drops below 2 mg/L¹ or any low DO concentration that negatively affects the behaviour and physiological processes of the organism². Aquatic environments exhibit patterns of spatial and temporal (daily and seasonal) variations in oxygen saturation³. Hypoxia can occur as a result of natural processes (e.g. seasonality) or anthropogenic influences^{2,4}. It has been shown earlier that hypoxia affects the reproduction⁵, weight gain⁶ and growth^{7,8}. In general, aquatic animals respond to hypoxia with a suite of physiological, behavioural, biochemical and molecular responses^{9,10}. Responses to chronic hypoxia are different and include the production of greater quantities of respiratory pigment, changing the structure of the pigment and in addition all hematological parameters to one with an adaptive higher oxygen affinity¹¹. In conditions of

substantial variations in oxygen availability, many fish rely on surface respiration or even use specific organs or tissues for gas exchange¹².

Given that oxygen availability is the main stressors that affects the generation of cellular reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), superoxide anion radical (O₂^{•-}), and hydroxyl radicals (•OH), it has long been argued if hypoxia is able to induce oxidative stress in aquatic animals. Evidence that ROS production is a consequence of fish exposure to hypoxia was provided^{13,14}. The electron transport chain and the NADPH oxidase system are the main sources of ROS in *L. Larimichthys crocea*¹⁵. Aerobic organisms possess antioxidant defense systems to regulate cellular ROS level including enzymatic: superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC1.11.1.6), glutathione peroxidase (GPx; EC 1.11.1.9). SOD catalyzes the dismutation of O₂^{•-} to H₂O₂ and water and represents the first line of defense against ROS of all aerobic organisms. Changes in SOD activity in response to

*Correspondence:

E-Mail: biljana.kukavica@pmf.unibl.org

exposure to different types of abiotic stress such as metals¹⁶, pesticides¹⁷ and hypoxia^{15,18}.

Popovo minnow (*Delminichthys ghetaldii*) (Fig. 1A) is an extraordinary fish which has shown remarkable adaptability to different environmental conditions. This small endemic cyprinid fish can only be found in springs and streams in fields Ljubomir, Dabar and Fatnica in Bosnia and Herzegovina, and Bacina lakes in Croatia. It is well adapted to karst habitats of eastern Herzegovina, characterized by considerable fluctuations of environmental conditions. During Autumn and Spring floods, Popovo minnows tend to emerge from the underground waters to surface estaveles. In this period, the spawning process occurs and lasts for several months. Withdrawal of the water to the underground basins brings them back to their underground habitat^{19,20}. In the present study, we examined the effect of moderate hypoxia on SOD activity and oxygen transport mechanism in Popovo minnows' erythrocytes. Due to the fact there is almost no information about Popovo minnow biochemical properties, for the first time we have performed the characterization of the erythrocyte SOD.

Materials and Methods

Popovo minnows were collected in the flood plain of Fatnica field (43°00'53"N;18°19'24"E) using nets with 18 mm mesh size. The treatment of fish was conducted according to European directive 2010/63/EU²¹ and the "Animal welfare act" ("Službeni glasnik Republike Srpske, Official Gazette of Republic of Srpska", No 111/2008)²². Fishes were randomly divided into two groups and blood samples from the control group were collected immediately. Experimental fishes were placed in aerated containers filled with adequate volume of water and transported to the laboratory. Acclimatization period in aquarium with dechlorinated water lasted 21 days at room temperature of 20-22°C and light/dark cycle 12/12 h.

Experimental group was held under conditions of mild hypoxia (DO 3.5-4.0 mg/L) for 3 days. During the treatment oxygen saturation rate in aquarium decreased from 94 to 65%. Blood was collected by cardiac puncture and transferred to EDTA-containing tubes. Following erythrocyte lineage parameters were measured: number of erythrocytes (RBC), hemoglobin concentration (Hb), Packed cell volume (PCV), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin concentration (MCHC) and MCH (Mean corpuscular hemoglobin). Hemoglobin concentration was measured spectrophotometrically using an assay kit (Sigma Diagnostics 527-A). Red blood cell count (RBC) was determined in haemocytometer²³. PCV was measured in pre-heparinized microhematocrit tubes in duplicate using hematocrit reader. Hematological indices were calculated based on haemoglobin concentration, PCV and RBC. We have also performed the characterization of the erythrocyte superoxide dismutase. Blood samples were centrifuged, and plasma and buffy coat were discarded. After the hemolysis, hemoglobin and membranes were removed using ice cold ethanol-chloroform mixture. The separation of the superoxide dismutases isoforms was done using native polyacrylamide electrophoresis (PAGE) with a 10% running gel²⁴ followed by specific staining²⁵. Different SOD isoforms were identified using specific inhibitors: 5 mM KCN and 5 mM H₂O₂. After electrophoresis, the gels were incubated for 30 min in the inhibitor solution, and then specifically stained for SOD activity.

Isoelectric focusing was performed using 7.5% (w/v) acrylamide mini gels with 3 M urea and 2% ampholyte (pH range 2-11) (Amersham Pharmacia Biotech) for 3 h at 4°C²⁴. Voltage was increased gradually: 90 min 100 V; 60 min 250 V and 30 min at 500 V. Phosphoric acid (10 mM) and NaOH (20 mM) were used as anode and cathode buffers. Before

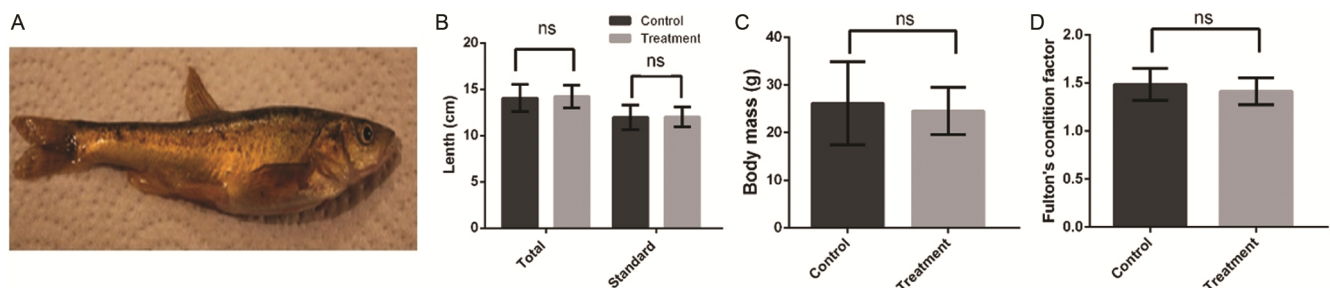


Fig. 1 — (A) Representative image of Popovo minnow (*Delminichthys ghetaldii*); (B) total and standard length; (C) body mass; and (D) Fulton's coefficient of the control group and the group exposed to mild hypoxia for three days. [ns, not statistically significant]

loading on the gel samples were mixed with loading buffer containing 40% glycerol and 20% ampholyte (pH range 2-11). SOD activity on the native gel was determined densitometrically using Total Lab software. The results were statistically processed in the program Graphpad Prism.

Results

All fish in the experimental group survived mild hypoxia for three days. In addition, mild hypoxia lasting 3 days did not lead to significant changes in morphometric parameters, mass and Fulton's coefficient in experimental group compared to control (Fig. 1 B-D). Significantly higher hemoglobin concentrations ($P < 0.05$) were detected in fish subjected to mild hypoxia (75.55 g/L) compared to control (70.23 g/L) (Fig. 2A). In addition, statistically significant differences were measured for MCH between control and treated samples ($P < 0.05$)

(Fig. 2B). No statistically significant differences were observed in the values of MCV, MCHC, PCV, RBC between control and treated samples (Fig. 2 C-F).

Native PAGE resolved the presence of three CuZn SOD which was demonstrated with inhibition with KCN and H₂O₂ (Fig. 3A). The contribution of the three SOD isoforms to the total SOD activity was different in both control and treated samples (Fig. 3B). Total SOD activity of experimental group was increased by 36% in comparison to control group (Fig. 3C). The SOD1 isoform made the largest contribution to the total SOD activity. In addition, the activity of SOD1 increased the most in conditions of hypoxia ($P < 0.01$). Two anionic SOD isoforms with pI values 3.4 and 3.5 were determined by isoelectric focusing (Fig. 4) in control and hypoxia-exposed samples.

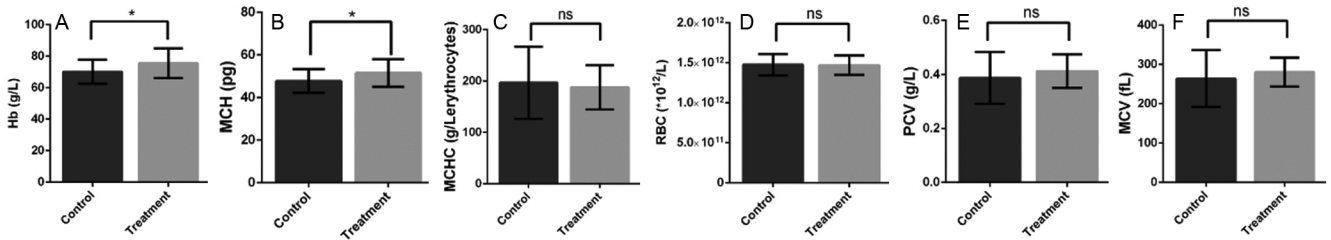


Fig. 2 — Hematological parameters of Popovo minnow control group and the group exposed to mild hypoxia for three days. (A) hemoglobin (Hb) concentration; (B) Mean corpuscular hemoglobin (MCH); (C) Mean corpuscular hemoglobin concentration (MCHC); (D) red blood cells (RBCs); (E) Packed cell volume (PVC); and (F) Mean corpuscular volume (MCV). [Asterix indicate statistically significant differences: * $P < 0.5$; *** $P < 0.01$]

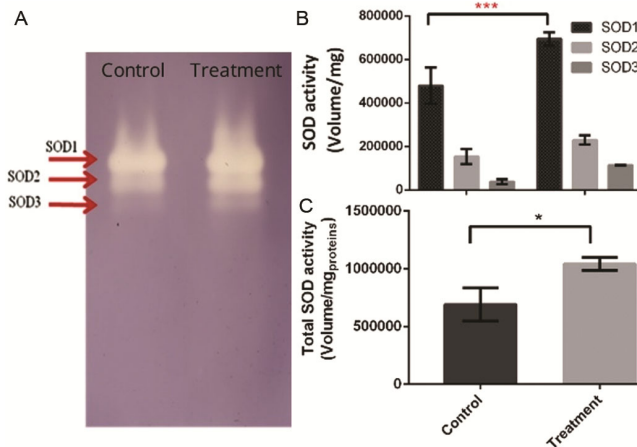


Fig. 3 — (A) Native gel with separated superoxide dismutase (SOD) isoforms: Lane 1 control samples, and lane 2 treated samples (exposed to mild hypoxia for three days). Arrows indicate different SOD isoforms; (B) Total SOD activity in control and treated samples; and (C) Activity of individual SOD isoforms in control and treated samples. [Asterix indicate statistically significant differences: * $P < 0.5$; *** $P < 0.01$]

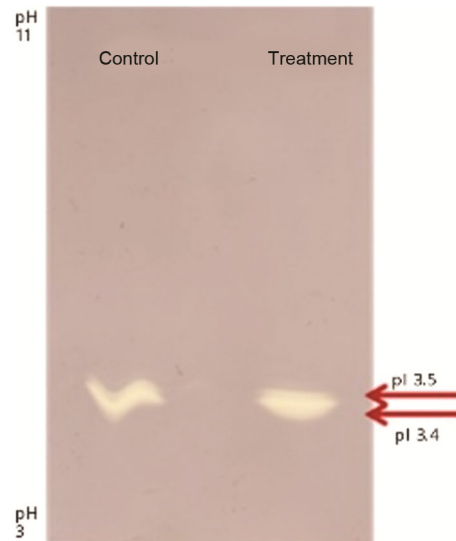


Fig. 4 — Superoxide dismutase (SOD) isoforms detected by isoelectric focusing on pH gradient (2-11). Lane 1 control samples, and lane 2 treated samples (exposed to mild hypoxia for three days). Arrows indicate different SOD isoforms.

Discussion

Aquatic environments exhibit wider extent of oxygen content fluctuations than terrestrial. Therefore, aquatic animals have evolved different responses (behavioural, physiological, ecological, biochemical) to counteract hypoxia²⁶. Popovo minnows exposed to mild hypoxia for three days successfully survived without significant changes in morphological parameters, mass, and Fulton's coefficient (Fig. 1). The karst area inhabited by the Popovo minnow is subject to frequent fluctuations in environmental conditions¹⁹. A possible innate ability to adapt enables the Popovo minnow to survive conditions of mild hypoxia for three days. Yellow catfish survival in hypoxia condition was above 98% during three days of exposure²⁷. Our study showed that conditions of mild hypoxia succeeded in initiating changes in oxygen transport mechanism. Interestingly, hemoglobine concentration and PCV values have increased, but there is no change in RBC. On the other side significant increase in RBCs at the initial hypoxic stage (1 h) was shown, and then returned to the control levels in largemouth bass after 24 h²⁸. In contrast, Hb significantly decreased after 4 h and then recovered to the control levels at 24 h of hypoxic exposure²⁸. An increase in RBC, Hb and hematocrit percentage during the first 24 h of hypoxia in yellow catfish (*Pelteobagrus fulvidraco* × *P. vachelli*) juveniles has been demonstrated, however, with the extension of hypoxia (96 h), there was a decrease in the measured parameters with the extension of the duration of hypoxia (96 h)²⁷.

Differences in the response of hematological parameters to hypoxia conditions may be a consequence of the duration and intensity of hypoxia, the examined fish species, and its developmental stage. Our results showed that MCV has increased in the treated group in comparison to control group, but observed changes in MCHC were not significant (Fig. 2). This brings us to conclusion that the increase of PCV is the result of enhanced hemoglobin synthesis and increase of erythrocyte volume. The rise in haematocrit following hypoxic exposure apparently reflects increased oxygen carrying capacity. It was previously shown that in conditions of hypoxia, erythrocyte swelling²⁹ occurs as well as an increase in the number of RBCs after 6 and 24 h from the beginning of hypoxia, but the number decreases with the extension of the duration of hypoxia³⁰. Main goal during hypoxia is maintenance of oxygen delivery

which is conducted by several mechanisms: stimulation of erythropoietin and rise in RBC²⁶, modulation of hemoglobin oxygen affinity³¹, an increase of hemoglobin synthesis³². During chronic hypoxic conditions, similar changes can be observed. They include the enhancement of respiratory pigment production and modulation of its structure aiming for higher¹¹.

Even though the exact mechanisms have not been established yet, many studies show hypoxia induced oxidative stress in several fish species: common carp *Cyprinus carpio*³³ rotan *Percottus glenii*³⁴, medaka (*Oryzias latipes*)³⁵. Enzymes that are part of the antioxidant defense system are expected to increase during hypoxic conditions. This can be observed in several studies on fish^{30,33} and turtles³⁶. Our experimental conditions of mild hypoxia have induced SOD activity indicating that hypoxia has ability to cause oxidative stress. Although it led to an increase in total SOD activity, hypoxia did not lead to changes in SOD isoenzyme profiles of Popovo minnow RBC. The same SOD isoforms were detected by native electrophoresis and IEF in control and mild hypoxia-exposed samples (Figs 3 & 4). We have detected the presence of three CuZnSODs in Popovo minnow's RBC where the activity of SOD1 isoform differed the most between control and hypoxia-exposed subjects (Fig. 3C). Five CuZnSOD isoforms were detected in rainbow trout RBC, whose expression depended on diet and the presence of antioxidants and highly unsaturated fatty acids (HUFA)³⁷. Liver cell extracts of fish (*Mugil cephalus*) collected from polluted environment showed induction of new CuZnSOD isozymes³⁸. The remarkable increase in SOD activity in condition of hypoxia in *L. crocea* was showed¹⁵. Hypoxia induced a different SOD response in liver and muscle compared to gills of largemouth bass²⁸. It was reported that SOD activity in *Phoxinus lagowskii* liver is significantly elevated under hypoxic stress³⁹. The SOD and CAT activities first increase then decrease in the gills and livers of *Micropterus salmoides* exposed to environmental hypoxia⁴. In gill and muscle tissue SOD activity increases under DO of 0.8 mg/L during 12 h of exposure⁴⁰. Like the hematological parameters, duration and intensity of hypoxia, species specificity and its development period can influence the intensity of changes in SOD activity. Hypoxia conditions lead to the production of ROS⁴¹, so it is extremely important for aquatic organisms to have a

well developed antioxidant system, including SOD activity. Literature data show that the response of SOD to hypoxia conditions can be different^{39, 42}. The different response of SOD can be influenced by the intensity of hypoxia, the type of organism and the type of organ in which it is measured.

Expected increase in both frequency and severity of hypoxia on both marine and freshwater ecosystems might have a substantial effect on biodiversity. Environmental hypoxia can disrupt the homeostasis in fish, which consequently causes reduced food intake, growth rate, fertility and survival^{8,43}. Endemic species inhabiting vulnerable inland freshwater ecosystems are the most susceptible to changing environments⁴⁴. Research of the physiological mechanisms underlying their environmental adaptability is therefore of pivotal importance for future preservation strategies.

Conclusion

With the above results, one can conclude that the cat fish Popovo minnow, *Delminichthys ghetaldii* responds to hypoxia by enhancement of oxygen carrying capacity and by triggering the superoxide dismutase (SOD) activity. Popovo minnow is portrayed by extraordinary way of life, often in conditions of food and light scarcity. Even small changes in the environment may trigger a response. Perhaps one part of Popovo minnow's adaptability is to wide ecological amplitude derives from complex and sensitive antioxidant system.

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Conflict of Interest

Authors declare no competing interests.

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