

BIOCHEMICAL EFFECTS OF ENVIRONMENTAL NITRITE
IN MATRINXÃ (*Brycon cephalus*)

Gilberto Moraes
Department of Genetics and Evolution, Federal University of São Carlos. Rod.
Washington Luiz, Km 235.
São Carlos, SP, Brazil. CEP 13565-905.
E-mail <gil@power.ufscar.br>

Ive Marchioni Avilez, Alexandre Eneas Altran
and Lucia Helena de Aguiar
Department of Genetics and Evolution – Federal University of São Carlos. Rod.
Washington Luiz, Km 235.
São Carlos, SP, Brazil. CEP 13565-905.

Introduction

Nitrite usually occurs in aquatic environments as a product of bacterial activity. It normally comes from oxidation of ammonia and this (nitrification) depends on the water aeration. Another source of water nitrite is the industrial wastes (Nikinmaa, 1992; Heckman et al., 1997). The fish-culture systems also increase ammonia and nitrite, followed by undesirable consequences (Hargreaves, 1998; Hagopian & Riley, 1998). The organismal disorder arisen from such environmental disturbances are particularly observed in fishes.

The prompt nitrite effect in fishes is observed at the blood level. Plasma can accumulate it (Shechter, 1972), working as a vehicle to spread it over the tissues. Within the red blood cells it oxidizes the hemoglobin-Fe²⁺ to Fe³⁺ yielding methemoglobin, unable to transport oxygen. This effect is supposed to result in tissue hypoxia (Cameron, 1971; Bath & Eddy, 1980; Doblender, et al., 1996, Vedel, et al., 1998) even in the presence of oxygen (functional hypoxia). The intensity of methemoglobin formation is dependent on the non-oxygenated level of hemoglobin (Jensen, 1990; Jensen, 1992; Willians, et al., 1993). Methemoglobin content varies among the species and depends of the nitrite levels and the exposure time. The increase of nitrite concentration increases lead to raise methemoglobin concentration (Schoore, et al., 1995). Usually, the fish plasma concentration of nitrite is higher than environmental one.

Environmental nitrite can cause physiological disturbs as decreases in total hemoglobin, hematocrit and RBC. This phenomena can be explained by erythrocyte hemolysis (Kundsén & Jensen, 1997).

In freshwater teleosts, nitrite exposure is followed by several osmoregulation responses as hyponatremia, hypochloremia (Jensen et al, 1987), branchial chloride cell failure (Gaino et al, 1984), and inhibition of chloride uptake (Willians & Eddy, 1986).

Nitrite exposed fish is completely recovered in clean water (Huey & Beitinger, 1981), and a couple of mechanisms involved in such process are proposed. The first is NADH-Methemoglobin reductase system (Diaforase I) reduces hemoglobin-Fe³⁺ to Fe²⁺ and it role in nitrite detoxification has been studied in fish (Scott and Harrington, 1985; Woo & Chiu, 1997). The nitrate synthesis, the more oxidized form of nitrite, is a second way of detoxification (Doblánder & Lackner, 1997) and catalase and citocrome oxidase-aa₃ is proposed to take a share in such process (Doblánder & Lackner, 1996). However, both mechanisms are still unclear and further studies should establish the role of those enzymes in fish detoxification of nitrite.

In this study the environmental nitrite effects hematological and osmoregulator response, the methemoglobin formation and NADH-methemoglobin reductase system will be investigated in the neotropical teleost *Brycon cephalus* (matrinxã).

Material and methods

Juveniles of *B. cephalus* (matrinxã) ranging 90 ± 5g (means ± SD) were obtained from the fish farm Aguas Claras, Mococa, SP, Brazil. The fish were brought to the aquaculture facilities of the Comparative Biochemistry Laboratory. Before the experiments, fish were equally divided in four tanks of 250L, covered with black plastic sheets, provided with well-aerated water. Quality of water was measure by APHA (1980) (pO₂ = 7.5 ppm, pH = 6.8 ± 0.2, temperature = 23 ± 1°C, conductivity = 74.3 ± 4.8 µS. cm⁻³, alkalinity = 37 mg/L of CO₃⁻, hardness = 28 mg/L of CO₃⁻, ammonia concentration = 0,01 p.p.m, nitrite concentration = 0 ppm). The indoor-experimental tests were performed in August.

Experimental design

Kept starved for 1 day, one tank nitrite free was the control. In a semi-static system (with water replace each twenty four hours), six fish per tank were exposed to 1, 2, and 3 p.p.m of NO_2^- by 48 h. After this, the fish were collected and anesthetized with MS 222. A blood sample was drawn from the caudal vein and divided into aliquots for further analysis.

Blood analysis

Microhematocrit was done with blood samples centrifuged at 12.000 g for 3 min in capillary tubes. Total hemoglobin was colorimetrically determined at 540 nm in samples containing 10 μL of blood into 2.0 mL of Drabkin solution. Methemoglobin was optically quantified at 563 nm as Matsuoka (1997). Red blood cells were counted under light microscope with a Neubauer chamber and the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC) were calculated.

One blood aliquot was centrifuged at 12.000 g for 3 min and the plasma was used to flame photometric determinations of Na^+ and K^+ , and optical determination of Cl^- at 480 nm (APHA, 1980) and NO_2^- at 520 nm (Shechter, et al., 1972).

NADH-Methemoglobin reductase system

One aliquot of blood was re-suspended into 0.9% saline solution and centrifuged at 1.000 g for 10 min. This procedure was repeated thrice and the cells were re-suspended into 0,04 mL of mercaptoethanol-EDTA solution and the erythrocytes were lysed by termal shock (with liquid nitrogen). This hemolysis was used as enzyme source. The enzyme assay was performed into a buffer solution 0.2M Tris-HCl pH 7.5, 1,2 mM 2,6-dichlorophenol indophenol, 6 mM NADH, and a suitable enzyme aliquot. The substrate consume was optically followed at 600nm and one unit equals a decrease in absorbancy of 1.0 per minute, in 25°C as Beutler (1984). The specific activity is expressed U (units) per mg of hemoglobin (U/mg total Hb).

Statistics

For comparison of the dates was used STATISTICA 5.5 software. Normality of the data set was evaluated by the SHAPIRO-WILL W test with 95% of

confidence limit. The parametric test ANOVA was used to compare the groups and the Post-Test of multiple comparisons DUNCAN was applied considering $p < 0.05$.

Results

Increasing concentrations of environmental nitrite affected the blood parameters of *B. cephalus* (Table I). The methemoglobin and the plasma nitrite increased very sharply keeping high values (figure 1).

Hematocrit decreased in all the nitrite exposures, however total hemoglobin and the red blood cell number did not change. The MCV, MCH and MCHC did not change too.

The NADH- methemoglobin reductase enzyme system was detected in the red blood cells of *B. cephalus*. That enzyme activity was found in all the fish and it was not affected by nitrite exposure (Table1). The figure 1 shows the trend of this enzyme during the nitrite exposure for 48 hours.

No significant effects were found in plasma protein, K^+ , Na^+ and Cl^- under nitrite exposure.

Table 1. Hematological parameters of *Brycon cephalus* exposed to environmental nitrite for 48h.

PARAMETER	NITRITE (ppm)			
	0	1	2	3
Total blood				
MetHb	0.17± 0	56.1* ± 6	84.3* ± 7	78.4* ± 8
Ht	35.8 ± 2	26.0* ± 3	24.0* ± 4	23.9* ± 2
Total Hb	9.06 ± 2	8.50 ± 2	7.35 ± 1	8.10 ± 1
RBC	2.40 ± 0.2	1.86 ± 0.5	2.00 ± 0.6	1.98 ± 0.3
MCH	37.96 ± 4.9	47.30 ± 4.8	39.02 ± 2.6	41.72 ± 4.1
MCV	133.6 ± 13	145.6 ± 14	127.4 ± 12	122.4 ± 6
CMCH	2.85±0.39	3.22±0.40	3.05±0.41	3.41±0.42
NADH-MetHb reductase	0.33±0.14	0.52±0.20	0.47±0.06	0.43±0.12
Plasma				
NO ₂ ⁻	0.01 ± 0.00	0.23* ± 0.02	0.34* ± 0.04	0.73* ± 0.05
Na ⁺	144.7 ± 12	130.5 ± 10	128.5 ± 12	103.0 ± 9
K ⁺	3.1 ± 0.5	2.7 ± 0.3	3.4 ± 0.3	3.2 ± 0.4
Cl ⁻	130.4 ± 6	134.2 ± 7	123.1 ± 12	110.6 ± 12
Protein	0.45 ± 0.04	0.49 ± 0.05	0.43 ± 0.04	0.41 ± 0.01

The values are expressed as: Ht (%), Total Hb (g.dL⁻¹), Red Blood Cells-RBC (10⁶.mm⁻³), Mean Corpuscular Hemoglobin-MCH (pg.cell⁻¹), Mean Corpuscular Volume-MCV (μmm³), mean corpuscular hemoglobin concentration –MCHC (%), NADH-methemoglobin reductase (U.mg total Hb⁻¹) Methemoglobin-MetHb (%), Na⁺ and K⁺ (mEq.L⁻¹), Cl⁻ (nmol.mL⁻¹), Protein (mg.mL⁻¹), NO₂⁻ (nmol.ml⁻¹). The mark (*) means significantly different (p< 0.05) as compared to the control.

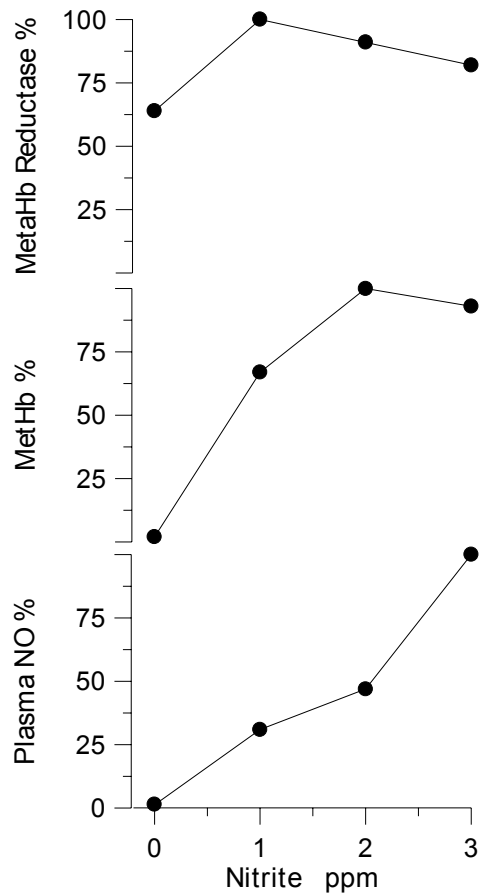


Figure 1. Relative concentration (%) of blood methemoglobin reductase, methemoglobin and plasma nitrate of *Brycon cephalus* (matrinxã) exposed to NO_2^- for 48 h, considered as 100% the maximum value of the parameter.

Discussion

Nitrite exposure in fish is supposed to result in tissue hypoxia that usually causes significant stress (Huey et al, 1980, Arillo et al, 1984, Hilmy et al, 1987). As a common classical response to stress from hypoxia, the increase of hematocrit should be expected. This strategy increases the number of red cells and the content of hemoglobin to keep the oxygen availability (Swift, 1981; Peterson, 1990). However, these responses were not detected in several fishes (Eddy, et al., 1983; Hilmy, at al 1987; Tucker, at al 1989; Jensen, 1990; Knudsen & Jensen, 1997; Woo & Chiu, 1997), or are thinly discussed. In this particular, decrease of the hematocrit of matrinxã can be attributed to the blood cell lyses (Jensen, 1990; Knudsen & Jensen, 1997), for the reduction of number of cells without changes of MCV, MCH and MCHC. This response should remove the blood methemoglobin but it will reduce the hemoglobin availability. Decrease of hematocrit in matrinxã without changes of the red blood cell number and total hemoglobin is suggestive of hemolytic anaemia, which is a posterior response to functional anemia (Scarano & Saraglia, 1984). Those authors propose the increase of methemoglobin as an early functional anaemia.

The NADH-methemoglobin reductase system has been detected in the most animals in nature, as well as in *B. cephalus*. This enzyme recovers the hemoglobin from methemoglobin keeping the equilibrium between both forms. Among the fishes, it was reported in the channel catfish *Ictalurus punctatus* (Huey & Beitinger, 1981), the rainbow trout *Salmo gairdneri*, *Oncorhynchus kisutch*, *Oncorhynchus nerka*, *Salmo malma* (Scott and Harrington, 1985) and *Lates calcarifer* (Woo and Chiu, 1997) and others.

Several studies attribute to the nitrite exposure the increase of methemoglobin concentration. Also, the recovery of the methemoglobin levels to normal values is observed as the fish return to nitrite free water. Schoore and col (1995) attribute this fact to the activity of NADH-methemoglobin reductase system, in spite of it was not assayed. One study on enzyme changes in fish exposed to nitrite is reported by Woo and Chiu, (1997) but no significant change was observed. The same occurred in matrinxã exposed to nitrite, as the level of NADH-methemoglobin reductase system did not change under any level of environmental nitrite. However, the presence of that system is very important for the species because it prevents the hemoglobin oxidation. The fact of NADH-methemoglobin reductase system be unchanged in matrinxã, does not

mean that this enzyme is not working, but more likely it was not induced as proposed to *Lates calcarifer* (Woo & Chiu, 1997).

As well as the nitrite attacks the heme group of hemoglobin it is possible that other heme proteins are affected, like the NADH-methemoglobin reductase system. This enzyme also presents a heme group in the molecular structure (citocromo b5). This attack could camouflage an increase of this enzyme production. In rainbow trout, Arillo and col. (1984) suggest that nitrite could attack hemoproteins as citocrome P450 in liver.

The main characteristic of fish nitrite exposure is the increase of methemoglobin and plasma nitrite concentration (Cameron, 1971; Shechter et al, 1972). These levels are usually very low and the increase depicts different trends. The increase of plasma nitrite concentration reveals an exponential tendency and the methemoglobin concentration reached a plateau. This characteristic suggests an equilibrium of methemoglobin formation by the reductase system activity (Huey et al, 1980).

The exposure of matrinxã to nitrite did not change the ion concentrations. In the marine teleost *Lates calcarifer* the enhancement of plasma sodium and chloride has been reported (Woo and Chui, 1997) and such fact should be associated to environmental seawater. Plasma potassium concentration is proposed to be associated to nitrite uptake. The increase of plasma K^+ in *Cyprinus carpio* exposed to NO_2^- is reported, and the direct correlation for both ions leads to such assumption (Jensen, 1990). In matrinxã, the plasma concentration of pattern nitrite followed the environmental one but the plasma level of potassium remained constant indicating that hipercalemia did not occur in matrinxã. The Cl^- concentration did not change in the most of the freshwater teleosts fish. However, nitrite uptake by chloride cell in gills occurs by competitive interaction between Cl^- and nitrite with the uptake sites. Gaino and col. (1984) suggest that there is no decrease of Cl^- concentration because the hipertrophia of some gill chloride cells. Other exchange mechanism may be an hyperactive response working jointly to chloride cells to maintain the physiological Cl^- levels, despite the nitrite competition or decrease of HCO_3^- production. This process could result in degeneration in these cells. That author showed that nitrite inhibits the carbonic anhydrase of the gills (Cl^- exchange with HCO_3^- in gills) *in vitro*.

The present data call attention to the fact that the anti-oxidative mechanism to prevent the hemoglobin conversion to methemoglobin in the freshwater teleost

matrinxã exposed to nitrite is not enough efficient. No other mechanism to prevent nitrite deleterious effects seems to work in matrinxã, since the external and the plasma concentration of nitrite was practically the same. Those fact plus the osmotic disturbs in matrinxã, are cumulative and certainly responsible for the great sensibility of the species to the nitrite poisoning.

References

- APHA 1980 Standard methods for examination of water and wastes. 12. ed., Washington, DC: Join Editorial board.
- Arillo, A., Gaino, E., Margiocco, C., Mensi, P. Schenome, G. 1984 Biochemical And Ultrastructural Effects Of Nitrite In Rainbow Trout: Liver Hypoxia As Of The Acute Toxicity Mechanism. *Environ. Res.* 34:135-154.
- Bath, R.N., Eddy, F.B. 1980 Transport Of Nitrite Across Fish Gills. *J. Exp. Zool.* 214:119-121.
- Beuteler, E. 1984 Red Cell Metabolism: Manual Of Biochemical Methods. 3. Ed., Grune & Stratton, Inc, 187p.
- Cameron, J.N. 1971 Methemoglobin In Erythrocytes Of Rainbow Trout. *Comp. Biochem. Physiol.*, 40a:743-749.
- Doblender, C., Lackner, R. 1996 Metabolism And Detoxification Of Nitrite By Trout Hepatocytes. *Biochimica Et Biophysica Acta*, 1289:270-274.
- Doblender, C., Lackner, R. 1997 Oxidation Of Nitrite To Nitrate In Isolated Erythrocytes: A Possible Mechanism For Adaptation To Environmental Nitrite. *An. J. Fish. Aquat. Sci.*, 54:157-161.
- Eddy, F.B., Kunzilik, P.A., Bath, R.N. 1983 Uptake And Loss Of Nitrite From The Blood Of Rainbow Trout, *Salmo Gairneri* Richardson, And Atlantic Salmon, *Salmo Salar* L. In Fresh Water And Dilute Sea Water. *J. Fish Biol.*, 23:105-116.
- Gaino, E., Arillo, A., Mensi, P. 1984 Involvement Of The Gill Chloride Cells Of Trout Under Acute Nitrite Intoxication. *Comp, Biochem. Physiol. A.*, 77(4):611-617.

- Hagopian, D.S., Riley, J.G. 1998 A Closer Look At The Bacteriology Of Nitrification. *Aquacultural Engineering*, 18:223-244.
- Hargreaves, J. A. 1998 Nitrogen Biogeochemistry Of Aquaculture Ponds. *Aquaculture*, 166:181-212.
- Heckman, C.W., Campos, J.L.E., Hardoim, E.L. 1997 Nitrite Concentration In Well Water From Poconé, Mato Grosso, And Its Relationship To Public Health In Rural Brazil. *Bull. Environ. Contam. Toxicol.*, 58:8-15.
- Hilmy, A.M., El-Domiaty, N.A., Weshana, K. 1987 Acute And Chronic Toxicity Of Nitrite To *Clarias Lazera*. *Comp. Biochem. Physiol. C.*, 86(2):247-253.
- Huey, D.W., Simco, B.A., Criswell, D.W. 1980 Nitrite -Induced Methemoglobin Formation In Channel Catfish Transaction Of The American Fisheries Society 109:558-562.
- Huey, D. W., Beitinger, T.L. 1981 A Methemoglobin Reductase System In Channel Catfish *Ictalurus Punctatus*. *Can. J. Zool.*, 60:1511-1513.
- Jensen, F.B., Andersen, N. A., Heisler, N. 1987 Effects Of Nitrite Exposure On Blood Respiratory Proprieties, Acid-Base And Electrolyte Regulation In Carp (*Cyprinus Carpio*). *J. Comp.Physiol.* 157b:533-542.
- Jensen, F.B. 1990 Nitrite And Red Cell Function I Carp: Control Factors For Nitrite Entry, Membrane Potassium Ion Permeation, Oxygen Affinity And Methaemoglobin Formtion. *J. Exp. Biol.*, 152:149-166.
- Jensen, F.B. 1992 Influence Of Haemoglobin Conformation, Nitrite And Eicosanoids On K⁺ Transport Across The Carp Red Blood Cell Membrane. *J. Exp. Biol.*, 171:349-371.
- Knudsen,P.K., Jensen, F.B. 1997 Recovery From Nitrite-Induced Methaemoglobinaemia And Potassium Balance Disturbances In Carp. *Fish Physiol. Biochem.*, 16(1):1-10.

- Matsuoka, T. 1997 Determination Of Methemoglobin And Carboxyhemoglobin In Blood By Rapid Colorimetry. *Biol. Pharm. Bull.*, 20(11):1208-1211.
- Nikinmaa, M. 1992 How Does Environmental Pollution Affect Red Cell Function In Fish? *Aquatic Toxicology*, 22:227-238.
- Peterson, M.S. 1990 Hypoxia –Induced Physiological Changes In Two Mangrove Swamp Fishes: Sheepshead Minnow, *Cyprinodon Variegatus Lacepede* And Sailfin Molly, *Poecilia Latipinna* (Lesuer). *Comp. Biochem. Physiol. A*, 97(1):17-21.
- Scarano, G., Saglia, M.G. 1984 Recovery Of Fish From Functional And Haemolytic Anaemia After Brief Exposure To A Lethal Concentration Of Nitrite. *Aquaculture*, 43:421-426.
- Schoore, J.E., Simco, B.A., Davis, K.B. 1995 Responses Of Blue Catfish And Channel Catfish To Environmental Nitrite. *J. Aquatic Animal Health*, 7:304-311.
- Scott, E.M., Harrington, J.P. 1985 Methemoglobin Reductase Activity In Fish Erythrocytes. *Comp. Biochem. Physiol. B*, 82(3):511-513.
- Shechter, H. Gruener, N. Shuval, I. 1972 A Micromethod For The Determination Of Nitrite In Blood. *Anal. Chim. Acta*, 60:93-99.
- Swift, D.J. 1981 Changes In Selected Blood Component Concentrations Of Rainbow Trout, *Salmo Gardneri*, Exposed To Hypoxia Or Sulethal Concentration Of Phenol Or Ammonia. *J. Fish Biol.* 19:45-61.
- Tucker, C.S., Francis-Floyd, R., Bebeau, M.H. 1989 Nitrite – Induced Anemia In Channel Catfish, *Ictalurus Punctatus* Rafinesque. *Bull. Environ. Contam. Toxicol.*, 43:295-301.
- Vedel, N.E., Korsgaard, B., Jensen, F.B. 1998 Isolated And Combined Exposure To Ammonia And Nitrite In Rainbow Trout (*Oncorhynchus Mykiss*): Effects On Electrolyte Status, Blood Respiratory Properties And Brain Glutamine/Glutamate Concentrations. *Aquatic Toxicology*, 41:325-342.

- Williams, E.M., Eddy, F.B. 1986 Chloride Uptake In Freshwater Teleosts And Its Relationship To Nitrite Uptake And Toxicity. *J.Comp. Physiol. B.*156:867-872.
- Williams, E.M., Glass, M.L., Heisler, N. 1993 Effects Of Nitrite-Induced Methaemoglobinaemia On Oxygen Affinity Of Carp Blood. *Enviorn. Biol. Fishes.*37:407-413.
- Woo, N.Y.S. Chiu, S.F. 1997 Metabolic And Osmoregulatory Responses Of The Sea Bass *Lates Calcarifer* To Nitrite Exposure. *Environ. Toxicol. And Water Qual.*, 12(3):257-264.