Red blood cell parameters and osmotic fragility curve of *Colossoma macropomum* (Pisces, Osteichthyes, Mileinae) in captivity

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ABSTRACT. In order to establish reference values for certain haematological parameters and osmotic fragility curve for the blood of *Colossoma macropomum*, blood samples were collected from 38 fish bred on farms in the state of Tocantins, Brazil. Red blood cell count, haemoglobin content and haematocrit were determined. Osmotic fragility curves were determined for 30 minutes under resting conditions at 30°C, incubated curves at 37, 30 and 27°C for 24 hours and incubated curves for 12 hours at 27°C. The haematimetric values obtained were: red blood cell count= 1.58 ± 0.08 $x10^{12}$/L; haemoglobin= 7.2 ± 0.32 g/dL; haematocrit= 34.0 ± 1.6%; MCV= 217.6 ± 13.2fL; MCH= 46.3 ± 2.6 pg and MCHC= 21.5 ± 1.4%. Osmotic fragility of fish erythrocytes was assessed according to haemolysis rate. Haemolysis began at 85.5 mM NaCl and total haemolysis occurred at 17 mM NaCl. Samples from incubated curve for 24 hours showed total haemolysis in all concentrations, regardless of temperature. In the samples from incubated curve at 27°C for 12 hours initial haemolysis was observed at 154 mM and total haemolysis at 17 mM NaCl.

Key words: erythrocytes, osmotic fragility curve, fish, *Colossoma macropomum*.

RESUMO. Parâmetros eritrocitários e curva de fragilidade osmótica de *Colossoma macropomum* (Pisces, Osteichthyes, Mileinae) em cativeiro. A fim de estabelecer valores de referência de certos parâmetros hematológicos e a curva de fragilidade osmótica do sangue de *Colossoma macropomum*, as amostras de sangue foram coletadas de 38 peixes, provenientes de fazendas do Estado do Tocantins, Brasil. Foram determinados o número de eritrócitos e o conteúdo de hemoglobina e hematócrito. A curva de fragilidade osmótica foi determinada com a incubação dos tubos por 30 minutos a 30°C, incubadas a 37, 30 e 27°C por 24 horas e incubadas por 12 horas a 27°C. Os valores hematimétricos obtidos foram: número de eritrócitos= 1,58 ± 0,08 $x10^{12}$/L; hemoglobina= 7,2 ± 0,32g/dL; hematócrito= 34,0 ± 1,6%; VCM= 217,6 ± 13,2fL; HCM= 46,3 ± 2,6pg e CHCM= 21,5 ± 1,4%. A fragilidade osmótica dos eritrócitos de peixes foi conduzida de acordo com os padrões de hemólise. A hemólise iniciou-se a 85,5 mM de NaCl e a hemólise total ocorreu a 17 mM. As amostras da curva incubada por 24 horas demonstraram hemólise total para todas as concentrações testadas, independentemente da temperatura. Nas amostras da curva incubada a 27°C por 12 horas a hemólise inicial foi observada a 154 mM de NaCl e a hemólise total em 17 mM.

Palavras-chave: eritrócitos, curva de fragilidade osmótica, peixes, *Colossoma macropomum*.

Fish, birds, reptiles and amphibians have elliptical nucleated erythrocytes circulating in their peripheral blood. Contrastingly mammals have anucleated red cells usually shaped as biconcave discs (reviewed by Campbell, 1991) and varying widely in number and size according to the species (Jain, 1986; Guerra-Shinohara, 1996).

The establishment of reference values for species may be helpful in the diagnosis of pathologies affecting blood cells (Heming, 1989), mainly in farmed species. One of the factors responsible for haematological changes in fish is stress caused by handling, transport, and captivity conditions, such as water temperature and pH (Heming, 1989; Campbell and Murru, 1990). In Brazil, where fish farming is an emerging activity, with increasing commercial fishing and rearing activities, the knowledge of fish physiology parameters is of

fundamental importance owing to the variety of fish species of high economic interest being farmed at present.

In haematological studies, the osmotic fragility test provides an indication of the ratio of surface area/volume of the erythrocyte. This test may be used in humans to diagnose hereditary anemia such as hereditary spherocytosis, in which there is an increase in osmotic fragility due to the presence of circulating spherocytes. The test may also be used in blood banks for screening through the use of a single point on the curve (61.5 mM) to investigate erythrocytes in blood bags (Dacie and Lewis, 1995).

In the case of human erythrocytes, the osmotic fragility curve may be determined by using the immediate reading method after 30-minute incubation, or after 24-hour incubation at 37°C (incubated curve) (Dacie and Lewis, 1995; Palek, 1995). The curve for 24 hour incubation at 37°C is useful to detect the presence of a small number of spherocytes in peripheral blood, since the immediate curve may appear normal, while in the 24 hour incubation the patient's curve departs from normality and shows greater fragility.

The osmotic resistance of erythrocytes differs among species (Oyewale, 1994a). The osmotic features of mammalian erythrocytes have been described in the literature. For instance, goats (order Artiodactyla) have erythrocytes with a mean corpuscular volume of 18.54 ± 1.84fL (Birgel, 1973) and are quite sensitive to a hypotonic solution (Perk *et al*., 1964; Jain, 1986). Camels have flattened, elliptical erythrocytes with a protein/lipid ratio of 3:1 in their membranes, which are quite resistant to osmotic lysis in hypotonic solutions (Perk, 1963).

Oyewale (1994b) studied the effect of changes in temperature and pH on the osmotic resistance of the erythrocytes of pigeons (*Columba livia*), peafowls (*Pavo cristatus*), lizards (*Agama agama*), and African toads (*Bufo regularis*). It was reported that at a temperature of 30°C and pH 7.6, the erythrocytes of pigeons and peafowls show sigmoid curves, with haemolysis beginning at a concentration of 85.5 mM NaCl. The point of 100% haemolysis occurred at a saline concentration of 51.3 mM in peafowls and only in pure water in pigeons. The osmotic fragility curves for lizard and toad erythrocytes showed higher fragility than those for pigeons and peafowls. The haemolysis rate in 85.5 mM NaCl was approximately 38.0, 12.0, 7.0 and 7.0% for erythrocytes of toads, lizards, pigeons, and peafowls, respectively.

The adaptation of methods and definition of reference curves for species have practical applications since they determine the points of initial and 100% haemolysis, and give inferences about the health status of individuals, or comparisons among species.

The objective of the present investigation was to establish reference values for a captive population of *Colossoma macropomum*, popularly known as tambaqui, which is being farmed on a large scale, has high nutritional potential as a protein source and is well accepted by consumers.

Material and methods

In current research 38 *Colossoma macropomum* (23 males and 15 females) were taken from fish farms in the municipality of Porto Nacional, state of Tocantins, Brazil. Mean total size was 29.98 ± 2.36 cm and weight 482.3 ± 136.91 g.

Fish blood (1.5 mL) was collected with a heparinized syringe by puncturing the caudal vein of the animal. Analyses for each fish were carried out after collection; samples were processed at a room temperature of 30°C, in the Human and Comparative Physiology Laboratory of the Biological Sciences Course at the University of Tocantins.

The number of erythrocytes (He) was obtained by using a blood dilution in Gower solution (1/200). Erythrocytes number was scored in a Neubauer chamber; haemoglobin concentration (Hb) was determined by the cyanomethaemoglobin method (according to Dacie and Lewis, 1995); haematocrit (Ht) was determined by blood sample centrifugation (5 min. 12,000 rpm) in microcapillary tubes. The haematimetric indices as mean corpuscular volume – MCV (Ht/He); mean corpuscular haemoglobin – MCH (Hb/He) and mean corpuscular haemoglobin concentration – MCHC (Hb/Ht) were calculated according to number of erythrocytes, haemoglobin and haematocrit.

Osmotic fragility curves were determined at room temperature (30°C) for 30 minutes and defined as immediate curves. Curves were also determined by incubation for 24 hours at 37, 30, and 27°C (according to Dacie and Lewis, 1995) and defined as incubated curves. Later, curves were determined by changing incubation time to 12 hours and fixing the temperature at 27°C. The NaCl solutions were prepared from a stock solution of 1.71 M NaCl, pH 7.0.

In order to check the concentrations of the NaCl solutions, a human erythrocyte fragility curve was determined concomitantly with the curves for erythrocytes of *Colossoma macropomum*.

NaCl concentration for initial haemolysis was estimated as the concentration with more than 5% of lysed erythrocytes, while the end of haemolysis was indicated when 100% lysis occurred.

Values obtained in the haematimetric analyses and in the determinations of osmotic fragility curves were compared between sexes by Student's *t* test.

Results

Table 1 shows the haematimetric values obtained for females and males of *Colossoma macropomum*. There was a significant difference in MCV between sexes.

Table 1. Distribution of the haematimetric values for the *Colossoma macropomum*. (x= mean; s= standard deviation; n= number of individuals tested; He= number of erythrocytes; Hb= haemoglobin concentration; Ht= Haematocrit; MCV= mean corpuscular volume; MCH= mean corpuscular haemoglobin; MCHC= mean corpuscular haemoglobin concentration)

n	Sex		He $X10^{12}/L$	Hb g/dL	Ht %	MCV fL	MCH pg	MCHC %
15	F	x	1.67	7.2	32	195.7	43.7	22.5
		s	0.28	1.1	4.9	29.2	7.6	4.5
23	M	x	1.53	7.2	35	233.1**	47.9	20.9
		s	0.21	0.9	4.8	40.4	8.0	3.7
Total		x	1.58	7.2	34	--	46.3	21.5
38		s	0.25	1.0	5.0	--	8.0	4.1

** $p<0.01$

Haemolysis in the erythrocytes of *C. macropomum* started at a concentration of 85.5 mM NaCl, and 100% haemolysis was observed at 17 mM NaCl (Figure 1). For human erythrocytes, haemolysis started to occur at concentrations of 85.5 mM and ended at 51.3 mM NaCl.

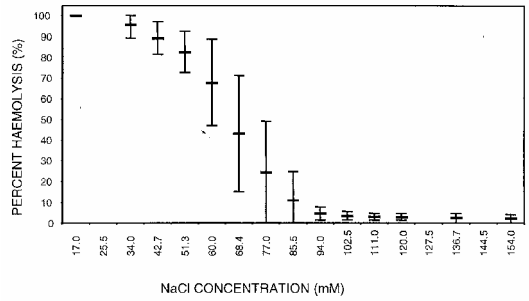

Figure 1. Osmotic fragility curve for *Colossoma macropomum* erythrocytes incubated for 30 minutes at ambient temperature (30ºC) (number of individual = 34)

When incubation time was fixed at 24 hours, there was total haemolysis of the erythrocytes kept at temperatures of 37, 30, and 27°C. Therefore, temperature was fixed at 27°C and the incubation time was reduced to 12 hours. The means and standard deviations for the osmotic fragility curve with incubation for 12 hours at 27°C are presented in Figure 2. In a 154 mM NaCl concentration there was already 13.7% haemolysis; total haemolysis occurred in a concentration of 17 mM NaCl.

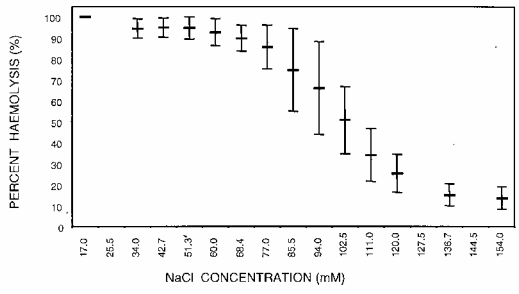

Figure 2. Representation of means standard deviations obtained for *Colossoma macropomum* at different NaCl concentrations after incubation at 27ºC for 12 hours (number of individual = 11)

The mean curves obtained for Colossoma erythrocytes after immediate readings and after incubation for 12 and 24 hours at 27°C are shown in Figure 3.

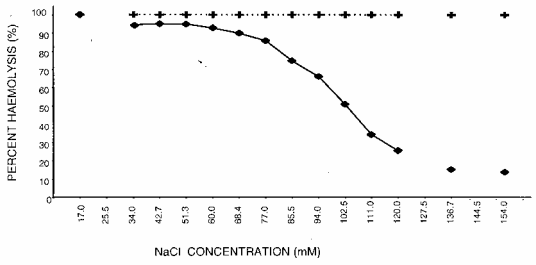

Figure 3. Osmotic fragility curve for different times, at 27ºC. (+ Incub. 24h; ♦ Incub. 12h)

Discussion

The use of farmed animals for experiments has the major advantage of being the easy way to take care of the animals and benefit from the knowledge of the environmental conditions under which they developed. However, results under these conditions cannot be applied to natural populations, mainly because of the environmental variables which interfere with the development of the animals and because of the genotype variation of the population.

In the present study specimens from fish farms near Porto Nacional were used. Therefore, the genetic variation was probably lower than that found in natural environments, mainly because of the source of the young fish. Since there is only one fingerling alevin production center in the area, the similarity among the animals tested may be high. It

is a fact that should be taken into account when analyzing results.

Haematology has been increasingly used as an aid for diagnosing the status of fish health and it can be useful for understanding fish disease (Heming, 1989). Lack of knowledge on the physiology of individuals and consequently of their patterns and their relationships to environmental variables is the main limiting factor in the use of this tool.

Physiological adaptive mechanisms used by fish in the presence of temperature variations, pH, oxygen concentration, ionic variations, and stress may alter haematimetric values (Heming, 1989; Nikinmaa, 1990; Schwantes and Val, 1996; Val, 1996). Fish may respond physiologically to reduction in oxygen levels by: 1) increasing the respiratory frequency, ventilation volume, and cardiac demand; 2) increasing the number of erythrocytes in circulation, haemoglobin concentration, and haematocrit; 3) increasing the oxygen affinity by adjustment of the Hb:NTP ratio (haemoglobin: nucleotide triphosphate); 4) the use of haemoglobins with different functional properties; and 5) reduction of metabolism (reviewed by Val, 1996).

Comparison of haematimetric values to detect intrinsic differences between sexes had no significant difference for tambaqui. Only MCV, which represents the ratio between haematocrit and number of erythrocytes, showed significant differences, suggesting that males have larger erythrocytes. However, this difference may also be attributed to the greater variation in the erythrocytes of males, as shown by a standard deviation of about 40%, which was higher than that of females (Table 1).

Aloni *et al*. (1977) showed that the lipoprotein membrane has sites for the action of temperature on the osmotic resistance of erythrocytes, and that differences in osmotic behavior may be related to the structural characteristics of the erythrocyte membrane. These investigators stated that the sites of osmotic rupture in human erythrocytes occur in regions of low cholesterol content.

There are some studies about the membrane proteins of the erythrocytes of mammals (Greenquist *et al*, 1978; Ballas *et al*, 1981, 1985, 1987; Smith *et al*, 1979; Guerra-Shinohara, 1996; Guerra-Shinohara and Barretto, 1999) comparing the erythrocyte size, its function on deformability and erythrocyte lysis. However it is not really so to fish erythrocytes due to the difficulty of methodology for membrane preparation from nucleated cells.

Nikinmaa (1990), demonstrated that mature nucleated erythrocytes appear to have protein filaments that form a marginal band of microtubules which permit greater resistance to deformation.

The immediate osmotic fragility curve obtained in the present study showed a sigmoid shape similar to that of human erythrocytes, although with different beginning and end points of haemolysis (Figure 1). It was comparable to those obtained in other vertebrates (March *et al*, 1966; Aloni *et al*, 1977; Kim and Isaacks, 1978; Isaacks and Kim, 1984; Oyewale, 1994a and b).

The Colossoma's erythroytes are more resistant to hypotonic solutions when compared to human erythrocytes. It is possible that this is an adaptive advantage for *Colossoma macropomum* in terms of maintaining cell volume and integrity during electrolytic disturbances caused by low water pH (Costa, 1995), or induced by hypoxia (Nikinmaa, 1990).

Comparison of the globular resistance between sexes showed no significant differences and suggests that a single reference curve may be defined for this species.

Kim and Isaacks (1978) studied the osmotic fragility of erythrocytes from the species *Osteoglossum bicirrhosum* (arawana), *Electrophorus electricus* (electric eel), *Pterygoplichthys* sp. (armored catfish), *Arapaima gigas* (pirarucu), and *Lepidosiren paradoxa* (lungfish). The erythrocytes of the electric eel and pirarucu showed a response similar to that of human erythrocytes when submitted to hypotonic NaCl solutions. However, the erythrocytes of the lungfish were quite resistant to haemolysis, showing 60% haemolysis in a solution of 17 mM NaCl. In the same study, these authors treated lungfish erythrocytes with distilled water for 15 minutes and for 1 hour and obtained 55 and 80% haemolysis, respectively.

Isaacks and Kim (1984) studied the osmotic fragility of *Neoceratudus fosteri* (Australian lungfish) and *Scleropages shneichardti* (an osteoglossid) and demonstrated that these species showed different globular resistances, ranging from 34 to 17 mM and from 94 to 60 mM, respectively. They compared their results with those obtained for *Osteoglossum bicirrhosum*, with haemolysis between 120 and 40 mM. The authors suggested that the globular resistance of fish might be related to lower permeability of the membrane to water and, in the case of pulmonate species, represents a possible adaptation to estivation conditions.

Heming (1989) studied the effect of emersion and manipulation on gas tension, blood acid-base

levels, biochemical parameters, haematocrit, and haemoglobin in nine fishes, by intra-aortic introduction of a cannula. This author did not count the number of erythrocytes before or after collecting blood samples. He observed an increase, albeit not significant, from 25 to 30% in the haematocrit values obtained before and after stress, respectively.

The physical and chemical characteristics of the nucleated erythrocytes of *Colossoma macropomum* differ from those of human erythrocytes, with cells unable to withstand incubation in hypotonic solutions for 24 hours, independent of temperature (37, 30, and 27°C).

Studies on the composition of the cell membrane, the function of the nucleus in maintaining osmotic fragility, and the influence of environmental factors on this species are necessary in order to understand its physiological and adaptive mechanisms.

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