ISSN- 0102-9010

PERIPHERAL BLOOD CELLS OF THE ARMORED CATFISH *Hoplosternum littorale* HANCOCK, 1828: A MORPHOLOGICAL AND CYTOCHEMICAL STUDY

Marcos Tavares-Dias[1] and José Fernando Marques Barcellos[2]

[1]Department of Physiological Sciences and [2]Department of Morphology,
Institute of Biological Sciences, Federal University of Amazonas (UFAM), Manaus, AM, Brazil.

ABSTRACT

Cytochemical studies are used to identify fish leukocytes and as a basis for studying the functions of these cells in cellular immune responses. In this work, we investigated the morphological features and cytochemical properties of the blood cells in the armored catfish (*Hoplosternum littorale*), a South American teleost. Reticulocytes, which accounted for 8-24.6% of the red blood cell population, stained with brilliant cresyl blue and contained a granular material similar to residual RNA. Thrombocytes, lymphocytes, monocytes, neutrophils, heterophils and eosinophils were identified and characterized in blood smears stained with May Grünwald-Giemsa-Wright. The lymphocytes were small, round cells with a basophilic cytoplasm and contained no periodic acid-Schiff (PAS), peroxidase or non-specific esterase activity. The thrombocytes were usually fusiform, with a hyaline cytoplasm that was acidophilic when stained with alkaline toluidine blue. The monocytes were round, with a basophilic and sometimes vacuolated cytoplasm that contained non-specific esterase activity. The neutrophils were large and round, with typical neutrophilic granules that sometimes showed moderate staining. The nuclei were rod-shaped and occasionally segmented, with PAS-positive granules that gave a weak reaction for peroxidase. The heterophils were large and round with coarse eosinophilic and basophilic, PAS-positive granules. The eosinophils were round and medium-sized, with eosinophilic granules that generally gave a negative reaction in all cytochemical stainings. The marked variation in the granulocyte morphology of *H. littorale* meant that a standard analysis based only on the morphology of these cells was insufficient for identifying all of the cell types.

Key words: Blood cells, cytochemical, freshwater fish, *Hoplosternum littorale*, leukocytes

INTRODUCTION

Although our knowledge of the blood cells of tropical freshwater teleosts has increased over the past decade, very little is still known about these species when compared to fish from temperate climates. *Hoplosternum littorale* (family Callichthyidae) is an armored catfish commonly known as tamoatá in Brazil. This species occurs in shallow, stagnant, oxygen-poor waters, such as the Paraguayan chaco, Guyana savanna, Venezuelan llanos and Amazonian flooded forest [1], and possesses optional aerial breathing in which the intestine is used as an accessory respiratory organ [1,2,22,23].

Some properties of the hemoglobin [22] and red blood cells [1] of *H. littorale* have been reported. In addition, Ranzani-Paiva and Eiras [15] reported the percentage of lymphocytes, monocytes, neutrophils, basophils, eosinophils and specialized granulocytic cells in this species from the Rio Paraná, but did not describe their morphological and cytochemical features. In other studies, the special granulocytic cells have been identified as reticular cells [12]. In studies of hypoxia in this species, Moura *et al*. [13] found only lymphocytes and neutrophils in blood smears, whereas others have reported circulating lymphocytes, monocytes, neutrophils and eosinophils [2]. These contradictory findings indicate that a standard analysis based only on morphological features is insufficient to identify the entire leukocyte population in *H. littorale*. Immature leukocytes can occur in circulating blood [16], especially granulocytes. Hence, cytochemical studies are needed to identify the leukocytes in this species and to provide a basis for investigations on the functions of these cells in cellular immune responses [16,17,20,21]. In the present work, we examined

Correspondence to: Dr. Marcos Tavares Dias
Departamento de Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade Federal do Amazonas (UFAM), Avenida General Rodrigo Octávio Jordão Ramos, 3000, Coroado I, CEP 69077-000, Manaus, AM, Brazil. Tel/Fax: (55) (92) 3647-4229. E-mail: mtavaresdias@ufam.edu.br; tavares-dias@bol.com.br

the morphological and cytochemical features of erythrocytes, thrombocytes and leukocytes in *H. littorale*.

MATERIAL AND METHODS

Fish and blood collection

Twenty healthy specimens of *H. littorale* (139-341.5 g and 20-25.5 cm long) were obtained from a commercial fish farm and transported to the Aquaculture Center at Universidade Estadual Paulista (UNESP), Jaboticabal, São Paulo state, Brazil. The fish were kept in 500 l tanks and acclimatized for 15 days. Caudal vein blood for the morphological and cytochemical analyses was collected into syringes containing 10% EDTA from fish anesthetized with benzocaine (1 g/10 l). Heparin was not used as an anticoagulant because it interferes with the staining of fish leukocytes, particularly the granulocytes [8,19].

Morphological and cytochemical analyses

For the morphological analyses, blood smears were stained with May Grünwald-Giemsa-Wright (MGGW) [18] or with 0.5% toluidine blue (pH 9.0). In the latter case, the smears were fixed in methanol for 10 min and then briefly washed in running water before being immersed in toluidine blue solution (0.5 g of toluidine blue and 1 g of sodium tetraborate in 100 ml of distilled water) for 1-2 min at room temperature. Finally, the blood smears were briefly washed in running water and air-dried.

For the cytochemical analyses, the blood smears were stained with periodic acid-Schiff (PAS) reagent [10] and also treated with salivary amylase as a control [7]; other blood smears were treated with ortho-toluidine hydrogen peroxide for peroxidase staining [6] or with naphthol AS-D esterase acetate [24] for non-specific esterase activity, with slight modifications of the original methods: the incubation time of the smears was changed to 60 min, which yielded better staining in this species, and nuclear staining was with Harris' hematoxylin instead of a solution containing fast red in aluminum sulfate because the latter solution did not stain the cell nuclei very well. Human blood smears were used as a positive control for these enzymes.

The metachromatic reaction was done with a solution of 0.2% toluidine blue, pH 3.2-3.4 (0.2 g of toluidine blue dissolved in 100 ml of distilled water containing 0.5 ml of acetic acid). The blood smears were fixed with lead subacetate (1 g of lead subacetate in 100 ml of 70% ethanol) for 10 min (modified from [11]). Immediately after fixation, the blood smears were again briefly washed in running water and immersed in toluidine blue solution for 50-60 min at room temperature. After a final brief wash in running water, the smears were air-dried.

Aliquots of blood were stained with brilliant cresyl blue (BCB, 1% in 0.65% NaCl) to identify and quantify reticulocytes, as described by Tavares-Dias [16]. In preparing the smears, equal parts of the blood sample and BCB solution (1:1, v/v) were mixed and incubated in a waterbath at 37°C for 20 min, followed by further mixing and preparation of the smears in the usual way. After air-drying, the smears were counterstained with MGGW and the reticulocytes were counted in 200 mature erythrocytes.

Most of the blood samples collected in syringes containing 10 µl of 10% EDTA showed a high degree of hemolysis, making it impossible to determine additional blood parameters such as the plasma glucose levels and red blood cell parameters (red blood cell count, hematocrit, hemoglobin concentration and others).

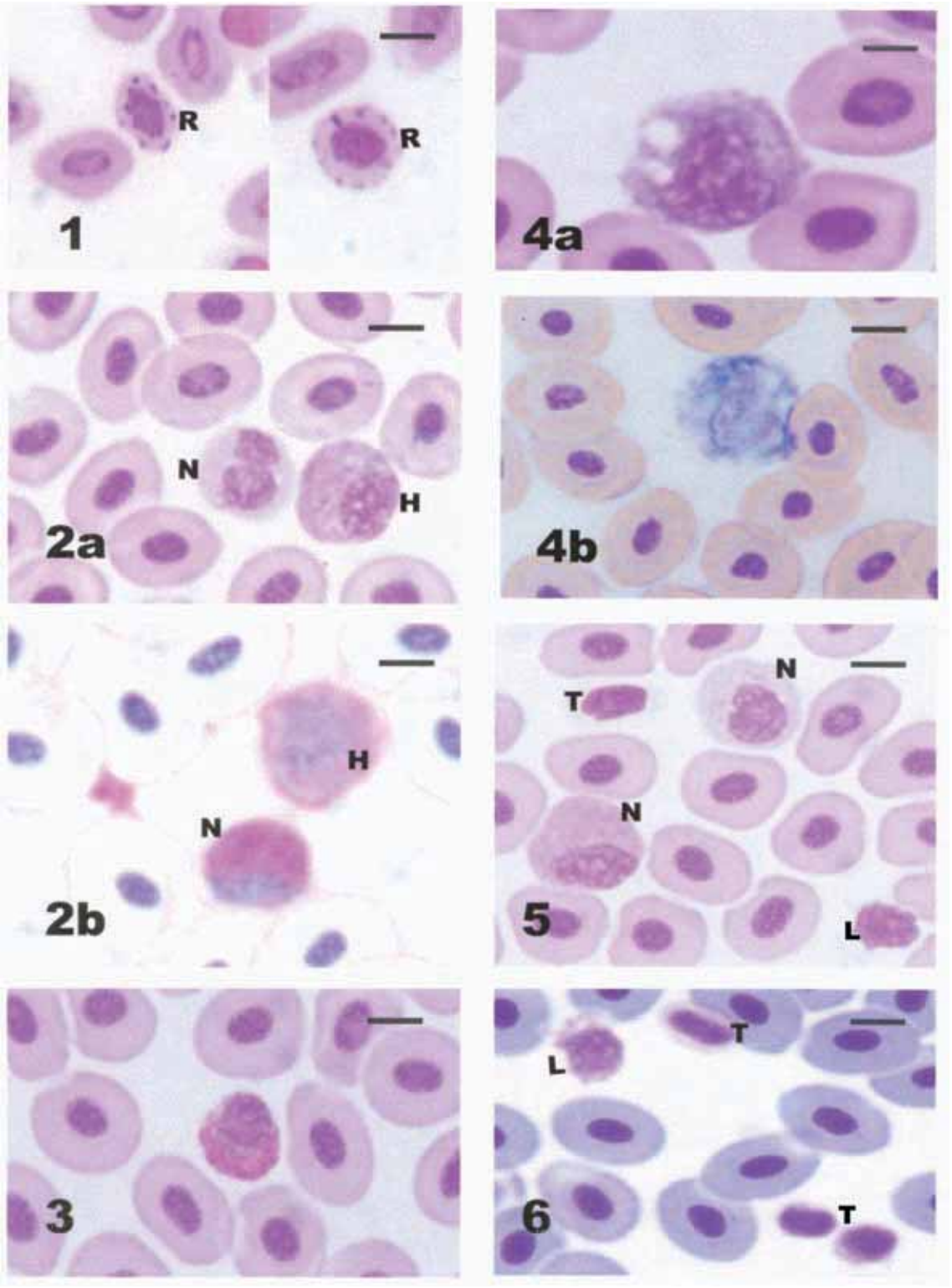
RESULTS

The mature erythrocytes of *H. littorale* had a predominantly elliptical form, with a nucleus that generally accompanied the format of the cells and a cytoplasm that was generally acidophilic by MGGW. Staining with BCB revealed a small network of basophilic ribonucleoprotein material in the cytoplasm, with the characteristics of immature cells; these cells were identified as reticulocytes and accounted for 8-24.6% (mean: 16.4%) of the red blood cell population (Fig. 1).

The morphological and cytochemical features of the thrombocytes, lymphocytes, monocytes, neutrophils, heterophils and eosinophils identified and characterized after staining with MGGW are summarized in Table 1 and illustrated in Figs. 2-6. The thrombocytes were fusiform and round, with extremely variable amount of glycogen granules. These cells were generally considered to be weakly PAS-positive. Neutrophils contained typical granules that were sometimes moderately stained. Granulocytes containing coarse eosinophilic and basophilic granules (Figs. 2A and 4B) were observed and the metachromatic reaction was negative. These cells were considered to be heterophils.

DISCUSSION

Fish blood contains erythrocytes of different sizes and shapes. The erythroblast stages are named based on the affinity of the cytoplasm for classic Romanowsky-type stains, which in turn is determined by the presence of hemoglobin [14,19]. *Hoplosternum littorale* erythrocytes stained with BSB showed small, dark, cytoplasmic structures characteristic of immature cells and were considered to be reticulocytes since they showed granular material equivalent to residual RNA. Similar findings were reported in other teleosts [4,5,14,19,25,26]. The characterization of these reticulocytes with BCB



Figures 1-6. Peripheral blood cells of the armored catfish *H. littorale*. **1.** Reticulocytes (**R**) stained with BCB. **2.** Segmented neutrophil (**N**) and heterophil **(H**) stained with MGGW (**2a**) and PAS (**2b**). **3.** Eosinophil stained with MGGW. **4.** Monocyte stained with MGGW (**4a**) and for non-specific esterase activity (**4b**). **5.** Round neutrophils (**N**) contained both typical and moderately stained granules. Note also the fusiform thrombocyte (**T**) and lymphocyte (**L**) stained with MGGW. **6.** Fusiform and rounded thrombocytes (**T**) and lymphocyte (**L**) stained with toluidine blue, both showing a light blue cytoplasm and purple nucleus. Bars = 4 μm.

in sodium chloride solution was easier and more reliable than in an alcoholic solution and allowed a larger area of the slides to be counted.

Hoplosternum littorale reticulocytes accounted for 16.4% (range: 8-24.6%) of the red blood cell population, compared to 11.96% in *Ictalurus punctatus* [5]. The reported values in other species include 8.7-13% in *Ictalurus nebulosus* [26], 1.6-3.4% in *Helostoma temmincki* [25], 2.9-9.5% in *Carassius auratus* and 31.1-48% in *Oncorhynchus mykiss* [14]. In contrast, in some marine and freshwater fish, this percentage is much lower (1-2%) [4]. The limited number of species examined to date and the considerable variation in the values reported make it difficult to establish baseline values for this parameter. However, *H. littorale* contained a larger number of mature than immature erythrocytes and this was directly proportional to the stage of cellular maturation; hence, these proportions can be used as an indicator of erythropoietic activity [5]. Since erythropoiesis in fish can be affected by anemia, temperature, seasonality and bleeding [5,16], this could influence the number of circulating reticulocytes. Consequently, the reticulocyte count may also be a useful indicator of the efficacy of a regenerative response after a reduction caused by anemia and environmental factors (temperature, oxygen availability, and so on).

Dividing red blood cells have been observed in most of the Brazilian teleosts studied so far [18]. In general, these cells normally occur in healthy fish and may vary among the species. Interestingly, *H. littorale* showed no cells of this type, perhaps because the spleen promptly removed senescent circulating cells from the blood. In healthy *C. auratus*, dividing red cells accounted for a mean of 0.2% of the erythron [14], a normal proportion that corresponds to senescent cells [5]. However, the incidence of these cells in division is much larger in fish with severe anemia, as reported during serious parasitism in *Leporinus macrocephalus* infected by the nematode *Goezia leporini* [9]. In healthy fish, dividing red blood cells stain normally but are hypochromic in infected specimens [9,19].

Considerable controversy has surrounded the leukocytes of tropical and temperate fish species [16,19]. Neutrophils are present in some fish whereas heterophils occur in others, while still others have both cell types [16,19]. *Hoplosternum littorale* contains thrombocytes, lymphocytes, monocytes, eosinophils, heterophils and neutrophils. The morphological features of the thrombocytes, lymphocytes and monocytes stained with MGGW agreed with the findings of Moura *et al*. [12].

Staining with MGGW revealed typical eosinophils and neutrophils, as well as neutrophils with extremely fine granules. The special granulocytic cells reported by Ranzani-Paiva and

Table 1. Morphological and cytochemical characteristics of thrombocytes and leukocytes in *H. littorale.*

Stains / Cells	May Grünwald-Giemsa-Wright stain	PAS	NSE	Peroxidase
Thrombocytes	Fusiform and round, with hyaline cytoplasm and fusiform nucleus	Weak	Negative	Negative
Lymphocytes	Round and small, with basophilic cytoplasm. The nucleus is round, being high its relationship with the cytoplasm	Negative	Negative	Negative
Monocytes	Round shape, with basophilic and sometimes vacuolated cytoplasm. The nucleus is generally eccentric and occasionally horseshoe-shaped	Negative	Positive	Negative
Neutrophils	Round and large, with the cytoplasm containing fine neutrophilic granules. The nucleus in small, rod-shaped, and occasionally segmented	Positive	Negative	Weak
Heterophils	Round and large, with the cytoplasm containing randomly distributed eosinophilic and basophilic granules	Positive	Negative	Negative
Eosinophils	Round and medium size, with a cytoplasm rich in eosinophilic granules	Negative	Negative	Negative

NSE = Non-specific esterase, PAS= periodic acid-Schiff.

Eiras [15] apparently correspond to an early stage of these neutrophils with extremely fine granules, and the eosinophils and basophils are possibly heterophils. No basophils with a metachromatic reaction were seen in the present work, although some heterophils with granules similar to basophils or eosinophils were seen.

Moura *et al.* [12] reported two types of granulocytes in *H. littorale* stained with May-Grüwald. One type had weakly stained basophilic granules with some purple granules, which these authors termed reticular cells. The other type of granulocyte had abundant, large, brown granules that were considered to be basophils. These basophils were easily ruptured during the preparation of smears and considerable care was needed to distinguish ruptured basophils from eosinophils. In another study, these same authors [13] reported only lymphocytes and neutrophils in *H. littorale*, with no monocytes. These contradictory results differ from the findings reported here, and this discrepancy may reflect problems during the preparation of the blood smears and a consequent difficulty in the identification of the leukocytes. In addition, the staining intensity of the heterophil granules of *H. littorale* with Romanowsky-type stains is variable and depends on the degree of cell maturation.

Cytochemical staining allows the identification of fish leukocytes and provides information on the functions of these cells [16,17,20,21], in addition to having a diagnostic value during pathological alterations [17,21]. The thrombocytes of *Scophthalmus maximus* [3], *Aristichthys nobilis*, *Astronotus ocellatus* and *Hoplias malabaricus* [16] are PAS-positive while in *Astyanax bimaculatus* they are negative [16]. The occurrence of glycogen granules in the thrombocytes of *H. littorale* varies with the stage of cellular maturation [16] and, as shown here, the PAS reaction was generally only weakly positive in these cells.

In *Oreochromis niloticus*, glycogen granules were observed in the cytoplasm of some lymphocytes, and monocytes, and in all of the neutrophils and thrombocytes [21]. In *H. littorale*, the typical neutrophils and those with extremely fine granules, as well as the heterophils, were PAS-positive, whereas the monocytes and eosinophils were negative. The presence of large stores of glycogen in leukocytes provides a reliable source of energy for cells that participate in phagocytosis, which explains the greater abundance of glycogen particles in the cytoplasm of professional phagocytes [16].

Esterase plays a role in the intracellular processing and/or trafficking of antigen, and the strong staining for non-specific esterase seen in fish monocytes indicates that they are involved in cellular defence mechanisms, particularly phagocytosis [16]. The monocytes of *H. littorale* were positive for non-specific esterase, in agreement with the findings reported for monocytes of *Carassius auratus* [27], *A. ocellatus*, *H. malabaricus* and *A. bimaculatus* [16]. In contrast, *S. maximus* [3] and *C. carpio* monocytes contain no non-specific esterase activity [20].

The presence of peroxidase in leukocytes is related to the efficient bactericidal system used as a defensive mechanism [16,21]. Ueda *et al*. [21] reported a positive reaction for peroxidase in neutrophils and eosinophils of *O. niloticus*. In *C. carpio*, only the neutrophils are positive for peroxidase activity [20]. As shown here, none of the *H. littorale* heterophils and eosinophils showed peroxidase activity, whereas the neutrophils exhibited low peroxidase activity. This difference in activity may reflect a higher proportion of immature neutrophils [16].

In conclusion, the morphological and cytochemical features of *H. littorale* thrombocytes and leukocytes were similar to those of other teleosts. The considerable variability seen in the granules of heterophils and neutrophils meant that the identification of these granulocytes required specific cytochemical staining. The information on the blood cells of *H. littorale* provided here should contribute to the standardization of hematological studies in this species.

REFERENCES

1. Affonso EG (2001) Respiratory characteristics of *Hoplosternum littorale* (Siluriformes, Callichthyidae). *Acta Amaz.* **31**, 249-262.
2. Affonso EG, Polez VLP, Corrêa CF, Mazon AF, Araújo MRR, Moraes G, Rantin FT (2004) Physiological response to sulphide toxicity by the air-breathing catfish, *Hoplosternum littorale* (Siluriformes, Callichthydae). *Comp. Biochem. Physiol.* **139C**, 251-257.
3. Burrows AS, Fletcher TC (1987) Blood leukocytes of the turbot, *Scophthalmus maximus* (L.). *Aquaculture* **67**, 214-215.
4. Catton WT (1951) Blood cell formation in certain teleost fishes. *Blood* **6**, 39-60.
5. Fijan N (2002) Composition of main haematopoietic compartments in normal and bled channel catfish. *J. Fish Biol*. **60**, 1142-1154.
6. Jacobs A (1958) Staining for leucocyte peroxidase. *Lancet* **1**, 697.

7. Lison L (1960) *Lipides et Lipoproteines*. Gauthiers Villars: Paris.
8. Mainwaring G, Rowley AF (1985) The effect of anticoagulants on *Blennius pholis* L. leucocytes. *Comp. Biochem. Physiol*. **80A**, 85-91.
9. Martins ML, Tavares-Dias M, Fujimoto RY, Onaka EM, Nomura DT (2004) Haematological alterations of *Leporinus macrocephalus* (Osteichthyes: Anostomidae) naturally infected by *Goezia leporini* (Nematoda: Anisakidae) in fish pond. *Arq. Bras. Med. Vet. Zoot.* **56**, 640-646.
10. McManus JFA (1946) Histological demonstration of mucin after periodic acid. *Nature* **158**, 202.
11. Mota I, Ferri AG, Yoneda S (1956) The distribution of mast-cells in the digestive tract of laboratory animals: its bearings on the problem of the location of histamine in tissues. *J. Microsc. Sci*. **97**, 251-257.
12. Moura MAF, Farias IP, Val AL (1994) Effects of temperature on leucocytes of *Colossoma macropomum* and *Hoplosternum littorale* (Pisces). *Braz. J. Med. Biol. Res*. **27**, 1589-1598.
13. Moura MAF, Oliveira MIS, Val AL (1996/1997) Effects of hypoxia on leukocytes of two Amazon fish *Colossoma macropomum* and *Hoplosternum littorale*. *Rev. UA. Ciên. Biol.* **1**, 13-22.
14. Murad A, Everill S, Houston AH (1993) Division of goldfish erythrocytes in circulation. *Can. J. Zool*. **71**, 2190-2198.
15. Ranzani-Paiva MJT, Eiras AC (1992) Células sangüíneas e contagem diferencial de leucócitos de 13 espécies de teleósteos do Rio Paraná- PR. *Simpósio Brasileiro de Aqüicultura, 7* e *Encontro Brasileiro de Patologistas de Organismos Aquáticos, 2,* Peruíbe, Brazil, October 27-30. pp. 173-182.
16. Tavares-Dias M (2006) A morphological and cytochemical study of erythrocytes, thrombocytes and leukocytes on four freshwater teleosts. *J. Fish Biol*. (In press).
17. Tavares-Dias M, Bozzo FR, Sandrin EFS, Campos-Filho E, Moraes FR (2004) Células sangüíneas, eletrólitos séricos, relação hepato e esplenosomática de carpa-comum, *Cyprinus carpio* (Cyprinidae) na primeira maturação gonadal. *Acta Scient.* **26**, 73-80.
18. Tavares-Dias M, Moraes FR (2003) Características hematológicas da *Tilapia rendalli* Boulenger, 1896 (Osteichthyes: Cichlidae) capturada em "pesque-pague" de Franca, São Paulo, Brasil. *Biosci. J.* **19**, 103-110.
19. Tavares-Dias M, Moraes FR (2004) *Hematologia de Peixes Teleósteos*. (Tavares-Dias M, ed). Ribeirão Preto.
20. Tripathi NK, Latimer KS, Burnley VV (2004) Hematologic reference intervals for koi (*Cyprinus carpio*), including blood cells morphology, cytochemistry, and ultrastructure. *Vet. Clin. Path*. **33**, 74-83.
21. Ueda IK, Egami MI, Sasso WS, Matushima ER (2001) Cytochemical aspects of peripheral blood cells of *Oreochromis* (Tilapia) *niloticus* (Linnaeus, 1758) (Cichlidae, Teleostei). Part II. *Braz. J. Vet. Res. Anim. Sci*. **38**, 273-277.
22. Val AL, Almeida-Val VMF (1995) *Fishes of the Amazon and their Environment: Physiological and Biochemical Aspects*. Springer-Verlag: Berlin.
23. Vaz MM, Torquato VC, Barbosa NDC (2000) *Guia Ilustrado de Peixes da Bacia do Rio Grande*. CEMIG/ CETEC: Belo Horizonte.
24. Wachstein M, Wolf G (1958) The histochemical demonstration of esterase activity in human blood and bone marrow smears. *J. Histochem. Cytochem*. **6**, 457.
25. Weinberg SR, Lobue J, Siegel CD, Gordon AS (1976) Hematopoiesis of the kissing gourami (*Holostoma temmincki*). Effects of starvation, bleeding, and plasma-stimulating factors on its erythropoiesis. *Can. J. Zool*. **54**, 1115-1127.
26. Weinberg SR, Siegel CD, Gordon AS (1972) The hematological parameters and blood cell morphology of the bullhead catfish, *Ictalurus nebulosus* (Le Suer). *Zoologica* **36**, 71-78.
27. Zinkl JG, Cox WT, Kono CS (1991) Morphology and cytochemistry of leucocytes and thrombocytes of six species of fish. *Comp. Haematol. Internat*. **1**, 187-195.

Received: October 13, 2005
Accepted: November 28, 2005