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Staining method for the study of blood cell types in a locally available fresh water fish, *Notopterus notopterus* (Pallas)

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Abstract

A clear understanding of the blood cell identification and their morphology is essential for each species of fish in order to establish Complete Blood Count (CBC) reference ranges. Hence, in the present study procedure followed for identification of cell types and their staining response with their morphology along with a detailed account of cell types in the blood of the locally available fresh water fish, *Notopterus notopterus* is undertaken and presented in this report. The blood samples were collected from the dorsal blood vessel which lies below the spinal cord and the blood immediately transferred to serum separator tube or a tube coated with heparin. The type of cells identified are, red blood cells (erythrocytes), White blood cells (leucocytes), agranulocytes, lymphocytes, monocytes, granulocytes, neutrophils, eosinophils and basophils. The condition of the fish was determined by assessing condition factor (K) by taking into consideration weight of the fish divided by length for assessing the stability of the fish and suitability of environment for survival growth and reproduction. The data indicated that the condition factor is 0.56 ± 0.01 with a range of 0.46 to 0.67. The K values indicated that the fish is in normal condition and healthy. This study provides comparable blood cells in fish as that of higher vertebrates.

Keywords: Fish, *Notopterus notopterus*, blood cells, staining technique

1. Introduction

It is well documented that ambient changes influence blood cell number, morphology and distribution (Srivastava and Choudhary, 2010) ^[14]. The haematological parameters are therefore widely used as an early signal of changes in fish health status, and have proven to be a valuable approach for monitoring the effects habitat changes on fish biology (Gabriel et al. 2004; Shaik and Ahmed, 2016) ^[5, 13]. In order to use blood parameters as biomarkers, it is necessary to know their standard values and reference interval for a given fish species and the ranges of normal values of the key haematological parameters are still undefined for some fish species living in different habitats (Parino *et al.* 2018) ^[86]. Haematological studies help in understanding the relationship of blood characteristics to the habitat and adaptability of the species to the environment. The blood cells of tropical fishes were more densely packed with haemoglobin (Hb) (Wells and Baldwin, 1990) ^[16] and had more total Hb due to increased number of red blood cells (Putman and Freil, 1978) ^[9] than less active species. Blood cells represent a free, connective tissue type cell, which may neither maintain intimate connections with other cells, nor possess intercellular substances constituting the homeostatic force of the organism (Kalashnikova, 1976) ^[6]. Haemopoietic tissues produce and subsequently release them into the plasma blood stream in which they are suspended. In teleost fishes, the anterior portion of the kidney, referred to as head kidney is a major organ of haematopoiesis, with minor sites including the spleen, liver and thymus (Lucy Towers, 2013) ^[7]. Erythrocytes are the dominant cell type in the blood of majority of fish species, it is widely accepted that fishes are like most other vertebrates. In fish, as in mammals blood cells including WBC are frequently used as indicators of fish health status because WBC are key components of innate defence leucocytes are involved in the regulation of immunological function in the organism (Ballarin *et al.* 2004) ^[2]. Since the fish, *Notopterus notopterus* is fast and continuous moving and active fish and it is well adapted in the local aquatic waters.

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After finding condition of the fish, the studies on cell types in the blood has been undertaken by applying specific staining techniques in identifying cells, their staining response and detailed account of their morphology which in this fish was not available particularly in this region.

Materials and methods

Notopterus notopterus a fresh water fish (Fig-2) selected for present study because of its sturdiness and easy availability in and around Kalaburagi (Gulbarga). Live specimens of the Indian fresh water fish *N.notopterus* were obtained from a local area in Kalaburagi (Gulbarga) Karnataka State, India. The size of the fish varied from 26.5 ± 1 cm in length and 105 ± 10 gm in weight. All sexes were used without discrimination. The fish were caught individually in a small hand net from the containers. After the preliminary investigation of the length and weight, the condition factor (K) was calculated thus. $K = (W/L^3) \times 10^2$, Where W = Weight and L = length. The fish were then placed belly upwards and blood samples obtained from the caudal circulation with the aid of a heparinized 2 cm disposable plastic syringe and a 21 gauge disposable hypodermic needle (Fig 1). The use of plastic syringe is a necessary precaution with fish blood because contact with glass results in decreased coagulation time (Smith *et al.*, 1952) [15]. The site chosen for puncture (about 3 to 4cm from the genital opening) was wiped dry with tissue paper to avoid contamination with mucus. The needle was inserted at right angle to the vertebral column of the fish and was gently aspirated during penetration. It was then pushed gently down until blood started to enter as the needle punctured a caudal blood vessel. Blood was taken under gentle aspiration until about 2 ml has been obtained. Thereafter the needle was withdrawn and the blood gently transferred into plastic containers. Blood has been separated in to two portions, one portion (Fig-3.) mixed with anticoagulant, samples (plasma) were then mixed gently and used for the measurement of haematocrite, haemoglobin concentration, and red blood cell count. From another portion of blood serum was obtained by centrifugation (Fig-3) and then used for the determination of biochemical parameters. All determinations were carried out in duplicates for each sample.

For making a film on clean glass slide (dry) transferred a drop of blood near the edge of the slide placed the spreader slide at the angle of 30° pulled back the spreader until it touches the drop of blood run along the edge of spreader. Push the spreader forward to the end of the slide with a smooth movement. Dry the blood smear at room temperature. Adequate drying is essential to preserve the quality of the film. Identification marking is made by using lead pencil or ball pen, number was written on the slide. If staining is to be done later, the blood smear must be fixing with methanol for 2- 3 minutes to prevent distortion of cells. Two staining solutions were used for staining of blood films. Leishman's stain: - Prepared by dissolving 0.15g of powdered stain in 100 ml of (acetone free) methyl alcohol. This stain is used in microscopy for staining blood smears as it provides excellent stain quality and is generally used to differentiate and identify leucocytes. It is based on a mixture of methylene blue and eosin. Leishman stain uses a methanol solution of staining dyes. 7-10 drops are applied/ After 5 minutes, 10-15 drops of a buffer solution (a Gurr buffer is used, pH 6.8) is added and mixed with the stain for 20-30 minutes, then washed off with the buffer solution. Giemsa stain:- 0.3g of giemsa staining

powder is dissolved in glycerol and kept at $56 - 60^\circ\text{C}$ (in water bath) for 2 hours. 25 ml of acetone free methyl alcohol is added and the prepared staining solution is kept at room temperature for one week. After completion of staining, the films are observed under oil immersion objective to study the morphology of the cells by taking photographs.

Results

The fish *Notopterus notopterus* (Fig-2) is available in the aquatic bodies of Kalaburagi. They can be collected by using caste net with the help of local fisherman. It is commercially important fish and it is gaining importance as food source in the local area. This fish is popularly called as "Chambari" by local people of Gulbarga. Among species available In Gulbarga area *Notopterus notopterus* is a prominent fish found in all types of habitats although there are many other fishes they are not uniformly available. The length of the fish ranged from 25.30 to 27.30 cm. and overall mean is 26.27 cm, weight of the fish is ranging from 95 to 115 gm and overall average is 102. 25 gm. The water temperature of the fish collected from the site and laboratory culture in the aquarium tank were measured and found to be 29°C . These values are considered as normal for the fish, *N.notopterus* as the fish comfortably swims and come to the surface for gulping air. The condition factor (K) determined for the fish *N.notopterus* in the present study by taking into consideration weight of fish divided by its length for assessing the stability of the fish and suitability of environment for survival growth and reproduction. The data indicates that the condition factor is 0.56 ± 0.01 with a range of 0.46 to 0.67 the K values indicates that the fish is in normal condition and healthy. The blood smears preparation after staining with Leishman's stain and observed under microscope the erythrocytes are clearly differentiated by their size and shape. The erythrocytes of the fish, *Notopterus notopterus* have oval shape. The erythrocytes located at the periphery seem to be large and matured. The nucleus is centrally placed and round or oblong in shape. (Fig-4). The number of erythrocytes (RBC) in the blood of fish *Notopterus notopterus* ranged from 1.17 -1.80 millions/ mm^3 with an overall mean of 1.33 million/ mm^3 . Although the fish white blood corpuscles have been well investigated, there is no unanimity regarding their classification. The fish leucocytes in peripheral blood are generally (a) Agranulocytes (b) Granulocytes (Fig-5). The nomenclature is based on affinity of acid and basic dyes and depends upon human hematology. The agranulocytes are identified based on presence or absence of granules in the cytoplasm. In the fish *N.notopterus* the agranulocytes have no granules in the cytoplasm. The most important distinguishing character is the presence of unlobed nuclei, thus they are distinguished from granulocytes, which possess specific segmented nucleus. Agranulocytes have two varieties (a) Lymphocytes (Fig-6), (b) Monocytes (7). The lymphocytes are most numerous types of leucocytes. The nucleus is round or oval in shape. They are rich in chromatin, although its structure is obscure, and is deep reddish violet in colour in preparation with Giemsa stain. The nucleus exhibits a dense chromatin and the cytoplasm has no visible granules. The main function of fish lymphocytes is to produce immune mechanism by the production of antibody. They constitute 43 to 50% of the total leucocytes in the fish *Notopterus notopterus*. Monocyte (Fig -7) consists of much less proportion of WBC population often absent in few fishes. It is suggested that they originate in the kidney and become apparent in the blood when foreign substances are present into

the tissue or blood stream. The cytoplasm is eosinophilic usually stains smoky bluish or pinkish purple. The nucleus of monocyte is fairly large and varied in shape. They found 0 to 1% of the total leucocytes in the fish *Notopterus notopterus*. In the fish *N.notopterus* the granulocytes are identified based on presence of granule in the cytoplasm. These cells possess specific granules in large numbers and they retain their nucleus. They are of three types (I) Neutrophils, (II) Eosinophils, (III) Basophils. The neutrophils are most numerous of the white blood cells. The fish *Notopterus notopterus* constitute high percentage 46 to 53% of total

leucocytes. The cytoplasm of these cells contains fine neutrophilic granules the nucleus is eccentric, rod shaped and occasionally segmented with compact chromatin. The eosinophils in *N.notopterus* are generally round and cytoplasm contains granules which have affinity to acidic dye and they take deep pinkish orange or orange red with purple orange background. The nucleus is lobed, takes deep orange purple or reddish purple stain. They found 1 to 4% of the total leucocytes in the fish *Notopterus notopterus*. Basophils are absent in the fish *Notopterus notopterus*.

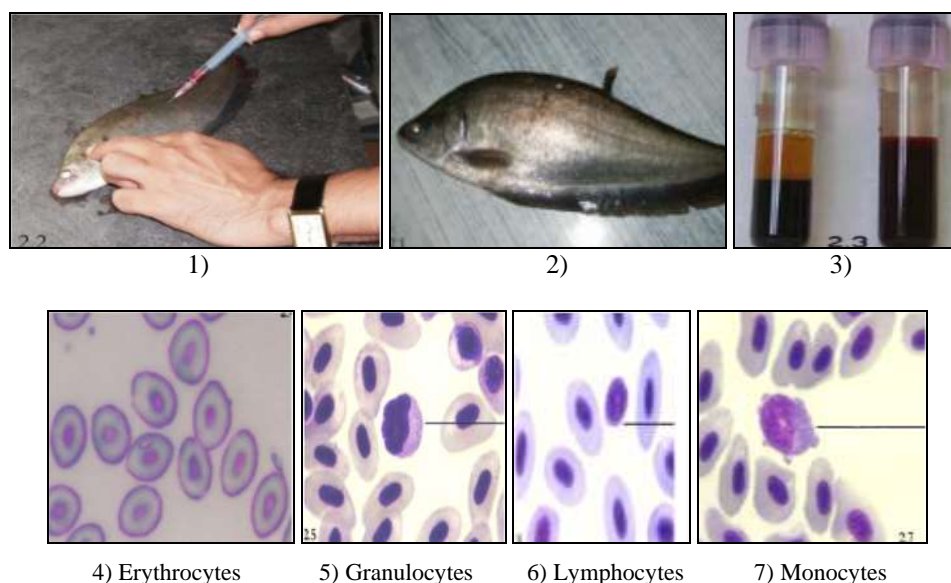


Fig 1-7: Showing the method of withdrawing the blood in the fish, *N.notopterus* & blood cells

Discussion

The fish, *Notopterus notopterus* selected for the present study were caught from local aquatic bodies and during the catch large numbers of fish were found to be available. This indicates that the water bodies from which fish, *N.notopterus* collected provides suitable ecology for its survival, growth and breeding activity. The fish thrives well in the aquatic bodies of Kalaburagi especially in the area having high vegetation which is submerged inside the water body which is favorable for its hiding and shelter a condition needed for spawning activity. The feeding of *N.notopterus* comprises of insects, their larvae, small fishes and weeds which has been identified based on their presence in the stomach after opening viscera. This fish can be considered as cornivorous preferring aquatic insects. The healthy condition of the fish is assessed in the present study, based on calculating condition factor by taking into consideration of the fish weight divided by its length. The value obtained by this calculation is 0.56 ± 6.01 with a range of 0.46 - 0.67 indicating healthy condition of the fish. Hematological parameters of fish are closely related to the response of fish to environmental and biological factors (Fernandes and Mazon, 2003) [4]. In response to ecological and physiological conditions, major changes occur in the fish blood composition, such as fluctuations in the levels of red blood cells (RBC), white blood cells (RBC), hormones, leukocyte counts and other basic components. Therefore, analysis of blood indices is a valuable guide an assessing the condition of fish, as it provides a reliable index of their physiological condition, a set of data that is especially important in fish aquaculture (Alyakrinskyaya and Dolgova, 1984) [1].

Hematological parameters can provide information on nutrient status, digestive function and routine metabolic level of fishes. Blood cells represent a free, connective tissue type cell, which neither maintain intimate connections with other cells, nor possess intercellular substances constituting the homeostatic force of the organism (Kalashnikova, 1976) [6]. Hematopoietic tissues produce and subsequently release them into the plasma bloodstream in which they are suspended. Erythrocytes are the dominant cell type in the blood of a vast majority of fish species, it is widely accepted that fishes are like most other vertebrates. The high erythrocyte number was associated with fast movement, predaceous nature and high activity with streamlined body (Rambhaskar and Srinivasa Rao, 1986) [10]. In the fresh water fish *N.notopterus* the lymphocytes are often small cells; but it is possible to find some large lymphocytes. Their nucleus occupies most of the cell and chromatin is compact and homogeneous and cell contains a paucity of basophilic-staining cytoplasm. A decrease in lymphocyte count especially of lymphocytes usually occurs in fish subjected to stress (Elsaesser and Clem, 1986) [3]. Heavy metal toxicity invariably reduces white blood cells count particularly lymphocytes (Witeska, 2005) [17]. In the present study the types of cells found are neutrophils which are round cells with an eccentric nucleus that can vary in shape from round to segmented, with the chromatin slightly compact; the cytoplasm is abundant and is lightly stained by eosin with observable fine clumps. Monocytes which are large cells of varying shape having also a large nucleus, occupying about two-thirds of the cell; the cytoplasm appears discreetly basophilic, vacuolated and lacking granules. Monocytes are immediate precursors of macrophages, when

they migrate to areas of acute inflammation with an enormous capacity for phagocytosis. Eosinophils are round shape and normally the nucleus is eccentric and the cytoplasm keeps large eosinophilic granules. Blood parameters are increasingly used as indicators of the physiological or sub lethal stress response in fish to endogenous changes. The possibility of evaluation depends on the availability of reference values as close as possible to normal values of the various blood components considered as reliable descriptors of healthy fish under natural conditions. Blood parameters are used in diagnoses as biomarkers. The changes in the hematological profile may indicate infestations and infections or even environmental changes (Ranzani-Paiva *et al.*, 1999) ^[11]. According to Serpunin and Likhatchyova, (1998) ^[12] the blood biochemical parameters of a fish species are true indicators of the state of health.

Conclusions

The blood cell identification and their morphology is essential for each species of fish in order to establish normal reference ranges and suitability of environment for fish survival and growth. The type of cells identified in the fresh water fish, *Notopterus notopterus* are similar to as found in higher vertebrates. Although the blood cells give differential staining response are active and viable. The condition of the fish was determined by condition factor (K). The K values indicated that the fish is in normal condition and healthy. This study provides comparable blood cells in fish as that of higher vertebrates.

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