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Use of Hematological Parameters as Assessment Tools in Fish Health Status

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Abstract

The purpose of this paper is to highlight the importance of blood analysis in assessing the welfare and health status of the fish. The blood is the circulating fluid that, through its dynamism, ensures the supply of all body cells with oxygen and nutrients. The peripheral blood is probably the most informative tissue because it may reflect all organism functions and can be non-lethally sampled in fish. Blood samples for hematological analysis are preferably collected by puncturing the caudal vessels or the heart, when there is no need for the fish to be sacrificed. Hematological indices, such as red blood cell counts, hemoglobin, hematocrit, leukocyte profile, differential white blood cell counts are widely known as indicators of several diseases and environmental stress in fish. The direct examination of the blood smears stained with May-Grunwald-Giemsa solution through routine hematological procedures can provide first signs of parasitosis (*e.g.* increase in eosinophil's number) or infections *(e.g.* increase in numbers of neutrophils, monocytes) together with the identification of some pathogens, such as haemoparasites and bacteria (septicaemia). Moreover, different observations of thin blood smears regarding the shape, size and color of the formed elements (red and white blood cell) have congruent diagnostic value. Our paper includes a broad overview of the working steps in hematological analysis and blood smear examination as well as the data gathered from our practice related to fish species variability manifestations under the action of extrinsic factors. As a conclusion, blood analyses may help as first indicators in the evaluation of fish health and valuable data base of blood investigations concerning their interspecies physiological reaction must be obtained from large numbers of individuals.

Keywords: hematological indices, blood smears, biochemical parameters, fish health

1. Introduction

The fish blood is the circulating fluid that, through its dynamism is involved in the basic functions, such as respiration, nutrition, excretion, defense and thermoregulation, ensuring the supply of all body cells with oxygen, nutrients and a large range of metabolites [8]. Many studies have demonstrated that fish are exposed to changes or pressures (stress factors), both in the natural environment (climate change, pollutants, anthropogenic impact) [14-16, 24], but also in an intensive aquaculture system (handling, transport, treatment, etc.) [16].

Because blood parameters can be influenced by a diversity of environmental stressors, they have the potential to be used as biomarkers. Everaarts et al., 1993 [45] has defined the biomarkars as a xenobiotically induced variation in cellular or biochemical processes, structures, or functions that is quantifiable in a biological system or sample. Studies on fish blood parameters are important to known factors regarding their physiologic capacity [14,

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41], being also a useful tool in the evaluation of their immune system [37].

Hematological parameters such as hemoglobin, hematocrit, red and white cell counts, erythrocyte sedimentation rates, and differential blood smears are widely used indicators of environmental stress; they may assessing the welfare and health status of the fish [3, 40].The hematological profile of fish is directly related to gender, gonadal development, nutritional status, seasonality, environment and dietary supplementation [34]. The changes seen in fish blood picture under certain pathological conditions can have specific and nonspecific characteristics. Specific characteristics provide direct diagnostics of given pathological condition of a species, whereas nonspecific characteristics only indicate existence of some changes in organism.

The blood oxygenation capacity is variable, reflecting changes in the physiological body's state and adaptation to environmental conditions. These changes can be made by efficient correction mechanisms of haematological parameters based on three main processes: (1) changing the production of erythrocytes (erythropoiesis); (2) release of a high number of erythrocytes from the blood forming organs into the circulation; (3) hydration or dehydration in blood plasma.

The ratio of circulating and stored blood is permanently changing, depending on the body's physiological needs (during exercise, digestion, absorption). The blood volume in fish has different values, depending on their evolution on the phylogenetic scale: 3-7% of the body weight in the bone fish, 4-8% in the cartilaginous fishes and 8-20% in the agnatha fish [26].

The peripheral blood is probably the most informative tissue because it may reflect all organism functions and can be non-lethally sampled in fish. With blood biomarkers, however, fish can be sampled and released without affecting population or community structure.

According to results obtained by different researchers [27, 28, 35] and our own experiences [5-7] blood analyses is a reliable method of assessment the physiological status. The purpose of this paper is to highlight the importance of blood analysis in assessing the welfare and health status of the fish.

2. Hematological analyses in fish: complexity and advantages

Fish blood sampling

The basic methods most usually used in fish hematology by establishing the hematological parameters (red and white cell numbers by chamber counting, haematocrit by centrifugation, hemoglobin by the cyanomethemoglobin procedure) are relatively simple and economically acceptable in terms of equipment and operating costs [15].

Blood samples for hematological analysis are preferably collected by caudal vessels or the heart puncture, when there is no need for the fish to be sacrificed. The site of blood sampling may influence some hematological parameters. Thus, if the blood is harvested from the dorsal aorta, the hematocrit value is higher than that of the ventral aorta harvest due to plasma filtration by the gills [14]. The filtered plasma is recycled to the heart via the venous system, and the blood harvested by caudal peduncle will have a slightly higher hematocrit, and the hemoglobin concentration will be higher than in the case of cardiac puncture harvest. Blood samples for analyses should be taken always at the same time of the day, because hematological status, as well as other physiological parameters has circadian rhythm.

To minimize the possible variations in the hematological values, the fish were caught gently in a small net, avoiding stress as much as possible and immediately anesthetized in MS222 in 1/15000 concentration [3]. The value of hematological indices may have will depend upon the accuracy or correctness with which they can be determined and the extent to which this information may be compromised during blood sampling and storage.

Because blood sample integrity degrades quickly during storage, before harvest, the blood is treated with anticoagulant, like EDTA or heparin lithium salt.

Examination of the erythrocyte and leukocyte profile

Haematological examination of fish, by quantifying the blood parameters and comparing the values obtained with known values under normal conditions for the species, has an important role in assessing the health status or metabolic disorders. Every species has characteristic number and size of red and white blood cells and distribution of leucocytes types (table 1, 2)

Hematocrit is a fairly accurate, simple and fast haematological variable, which is the percentage expression of the ratio between red cell mass and blood volume. Hematocrit is a test that can provide multiple information on some pathological conditions (assessment of anemia and polycythemia, increase in erythrocyte count), but it is also used in the calculation of erythrocyte constants. Allen (1993) [1] highlighted that "hematocrit is one of the primary diagnostic parameters for fish health". Hypoxia ("in vitro") can lead to erythrocytes swelling and, implicitly, to increased hematocrit value. Since erythrocytes have an intrinsically high rate of oxygen consumption, extending the rotation time of microhematocrit tubes may occasionally result in higher hematocrit values [14]. Hematocrit reading will be done immediately in order to prevent errors due to evaporation of the plasma tubes.

The hemoglobin concentration is a synthetic high value indicator for assessing the physiological integrity of fish. The change in hemoglobin concentration over the physiological limits, are a practical importance that can define anemia, hemodilution, nutritional disorders (decrease below normal values) or stress, hemoconcentration or dehydration (increase over normal values).

From the hematological indices are calculated a group of secondary indices known like mean corpuscular values (red cell absolute values). Thus, mean corpuscular volume (MCV) is the expression of the average volume of individual erythrocytes calculated with the following formula: MCV= (PVC x 10)/RBCc (fl). Mean corpuscular hemoglobin (MCH) is the expression of the average hemoglobin content of a single erythrocyte calculated with: MCH= (Hb x 10)/RBC (pg). Mean corpuscular hemoglobin concentration is the expression of the volume within the erythrocyte occupied by the hemoglobin and is calculated with: MCHC= (Hb x 100)/PVC (g/l) [5].

The number of erythrocytes in fish is also dependent on various internal factors such as age, sex, reproductive status or ploidy. Thus, the number of red blood cells and the hemoglobin concentration have a pronounced intraspecific specificity; there are species in which these parameters have extremely low values (0.5 - 1.5 mil erythrocytes/µl, 50 - 70 gl^{-1}Hb), respectively extremely high (3-4.2 mil/130 gl^{-1}). One of the main causes of these differences is the natural motility of the species: migratory pelagic species have a higher blood oxygenation capacity, whereas in low-activity benthic species the blood has a low amount of respiratory pigment and erythrocytes number [20, 32].

Lay and Baldwin (1999) [22] discover an inverse relationship between erythrocyte size and aerobic swimming ability in teleost fishes. They justify that a higher ratio of surface area to volume in smaller cells results in a shorter diffusion distance and allows faster oxygen transfer. In aquatic environment, O_2 availability may considerably fluctuate, and fish answer to hypoxic conditions with raising of blood oxygen carrying capacity – a rapid release of stored cells from a splenic reservoir or an increase in erythropoietic rate [16].

The fish size erythrocytes are characterized by high inter-specific variability; these dimensions differ according to phylogeny.

Thus, the cartilaginous fish have a large red cells, but fewer in number than the erythrocytes of the teleosteen fish.

Table 1. Red blood cell parameters in some fish species (*[3–4]– Grant 2014; [1]–Shaluei et al., 2012; [2-] Khara, et al., 2013; [6]- Shah, 2010*) [11, 21, 29, 30]

	RBC (x 10^6cel/µl)	***Hct (%)***	***Hb (gl^{-1})***	***MCV (fl)***	***MCH (pg)***	***MCHC (gl^{-1})***
Huso huso[1]	0,635±0,02	17,49±0,4	6,31±0,4	294,33±3,8	102,07±2,2	35,51±1,2
Acipenser stellatus[2]	1,36±0,86	6,55±0,46	26,6±0,86	517±11	126±0,22	24,5±0,34
Acipenser brevirostrum[3]	0,65-1,09	24-46	5,7- 8,7	307- 520	65,9-107,1	15- 30
Cyprinus carpio[3]	1,59-1,75	32 - 35	7,84-8,56	196,5-207,5	49,1	0,24
Carasius aurasius[4]	0,8-2,4	21 - 23	6,45-6,95	134,4-139,6	40,6-43,4	0,3
Tinca tinca[6]	0,9-2,1	19-30	4,8– 9,1	149,9–216	40,8–50,98	21,9–27,3
Oreocromis Niloticus[5]	1,9-2,83	27 - 37	7-9,8	115-183	28,3-42,3	22- 29

Table 2. White blood cells values in some fish species (*Le– leukocyte; L- lymphocyte; M– monocyte; N– neutrophile; Eo– eosinophile; B- basophile*) *[1]Tavales, et al., 2007; [2]Hrubec, et al., 2000; [3]Hunn, et al., 1992; [4,5]Groff, et al 1999; [6]Docan, et al, 2012* [6, 12, 17, 18]

	Leukocyte (x 10^3cell/µl)					
	Le	***L***	***M***	***N***	***Eo***	***B***
Ictalurus punctatus [1]	8,9-124	1,4-23,6	0,7-14,7	4,5-80,8	0	0-7,1
Oreocromis niloticus [2]	21,6-154	6,8-13,64	0,4-4,3	0,5-9,9	0-1,6	0
Oncorhynchus mykiss [3]	21	18,8	0,6	1,6	0	0
Cyprinus carpio [4]	37,8±2,88	32,26±35,15	0,19±0,76	1,13±3,78	0,19±0,38	0
Carassius auratus [5]	52,3±4,88	26,7±2,89	0,2±0,1	2,3±0,56	0,1±0,1	0
Acipenser baeri [6]	20,46±3,05	16,19±3,4	0,29±0,05	3,27±0,04	0,71±0,11	0

Different authors reported that toxic substances might cause morphological anomalies in erythrocytes, including nuclear anomalies, cell deformation, amitosis or hemolysis [31, 39, 43]. Due to their sensitivity to xenobiotics, fish erythrocytes are often used to evaluate genotoxic potential of pollutants or other adverse environmental factors. Aquatic pollution may also induce amitotic divisions of erythrocytes. Cellular anomalies indicate both external and internal damage of erythrocytes.

Houston (1997) [14] proposes the use of an erythron profile: an estimation of the relative abundances of red blood cells in various developmental stages. These stages include immature cells, intermediately developed cells, and mature cells, as well as dividing cells, enucleate cells, and karyorrhetic (degenerating) cells. Fish red blood cells are released from erythropoietic sites (spleen and head kidney) into adjacent capillaries at an early stage in their development, and complete their maturation within the circulation [10].

Different forms of stress stimulate the release of erythrocytes into circulation, thus which, the proportion of immature red cells in circulation can be used as an indicator of environmental stress.

The changes in erythrocyte parameters are used for both diagnosing presence and type of anemia and physiological adaptive mechanisms [33]. Anemia is a common pathophysiological response of fish to various adverse environmental impacts such as pollutants, pathogens or malnutrition and may affect both wild and aquaculture fish. Anemia results in reduced oxygen supply to the tissues and thus impairs energetic performans which may lead to reduced growth and reproductive ability, and imparment of health status. It may be caused by accelerated hemolysis impaired erythropoiesis or blood loss. Analysis of complete set of red blodd parameters (Hb, Ht, RBC, MCV, MCH and MCHC) accompanied by morphological evalation of erythocytes of blood smears allows to detect anemia, evaluate its severity determine of mecanism of mechanism of anemia development and compensatory power of organism [44]. According to Vosyliene (1999) [38, 39], the compensatory reactions increasing oxygen carriyng capacity of fish blood include: swelling of erythrocytes, syntessis of hemoglobin by circulating cells, amitotic division of erythocytes, releases of erythocytes from splen and head kidney reservoair, and activation of hematopoiesis.

Erythrocyte sedimentation rate (ESR) is a common hematological test can provide valuable information in case of tissue damage under stressful conditions it is also a non-specific method of evaluating the inflammation. ESR is a non-specific indicating the presence and intensity of a disease process. Increasing or decreasing the ESR value indicates physiological dysfunctions of the fish. Stress can increase ESR due to erythrocyte fragility [5-7].

The direct examination of the thin blood smears stained with May-Grünwald-Giemsa solution (Pappenheim) through routine hematological procedures help to identifying the stage of erythrocyte development and different types of leucocytes. Blood smears are prepare immediately and airdried, fixed in 95% methanol for 5 min then stained with Giemsa. Giemsa-stained blood smears are use for the measurement and assessment of blood cells. Blood cell dimensions may be determined by using a ocular micrometre. The fish erythrocytes are elliptical, and as such, two different diameters are provided: erythrocyte lengths (EL) and erythrocyte widths (EW). Erythrocyte and nuclear sizes (ES and NS) can be calculated according to formulas [(EL× EW×π) /4] and [(NL×NW×π) / 4], respectively [23]. White blood cells differentiation is based on shape and size of cells, color and relative size of nucleus and cytoplasm, presence and type of cytoplasmic granules [9].

Blood cells including WBCs are frequently used as indicators of health status in fish because WBCs are key components of innate immune defence and leukocytes are involved in regulation of immunological function in the organisms [2].

To determine leukocyte ratios, 100 total leukocytes are counted per slide, and data are recorded as percent neutrophils, lymphocytes, and monocytes. Changes in the leukocyte series typically occur in response to different aquatic stresses or may be correlated with the pathogenesis of various diseases. Numerous researchers have harness on the leukocyte stress response in fish as a tool for understanding the physiological effects of exposure to different contaminants in a high range of fish species [4, 25, 42]. In fact, they consider changes in the differential leukocyte count to be one of the most sensitive indicators of acute stress in fish [40], lowered WBC counts are an indication of acute stress in all fishes.

Lymphocytes are usually the most commonly present leucocyte type in some fish, accounting for as much as 85% of the total leucocyte population, excluding thrombocytes [12]. Lymphocytes are one of the most important cells to impact on a fish's immune response.

These cells produce antibodies by specific immunity and increasing macrophages. An increase of such immune cells can promote a fish's defence to an adverse condition [19]. The direct examination of the blood smears stained with conventional techniques can provide a first signs of parasitosis (*e.g.* increase in eosinophil's number) or infections *(e.g.* increase in numbers of neutrophils, monocytes) together with the identification of some pathogens, such as haemoparasites and bacteria (septicaemia).

3. Case studies

Our experimental studies on reaction of fish hematological status to action of some pathogens, highlights the importance of using hematological parameters as tools for assessing fish health. So, in case of European catfish raised into a flow-through system of the pilot aquaculture station from our Aquaculture Department, fed with two different feeds (41 and 46 % protein content - BP), it was observed that fish fed 41% BP did not react to external stimuli and the appetite decreased. The biochemical analysis of the feed revealed the presence of the toxic metabolites (aflatoxin B) secreted by the mold *Aspergilius flavus.* Physiological stress induced by toxic metabolites secreted by the mold is reflected in the hematological parameters. Decrease in hematocrit, hemoglobin and red blood cell counts are signs of catfish anemia. The leukocytic and thrombocytic lines were the most affected ones, whose modifications materialised in a leukopenia and thrombocytosis. Thus, the leukocytes significantly decreased accompanied by lymphopenia, accentuated neutrophilia, and eosinophilia (an increase in the number of eosinophils, suggesting a delayed sensitivity reaction). Neutrophilia was accompanied by an increased number of young neutrophils, which proves a "regenerative reaction" specific to the initial stage of neutrophilic fight.

A similar situation was reported in the rainbow trout infected with *Aeromonas salmonicid. H*ematological analyses of infected fish showed drastic changes of their hematology*:* decrease of the erythrocytes number, hemoglobin and hematocrit, as well as identifying of the poikilocytes and schizocytes on blood smears (figure 1). These quantitative and qualitative changes have led to fish anemia.

Investigating of the leukocyte profile of infected trout, highlights an increase in white blood cell counts as an attempt to strengthen cellular defense mechanisms. This leukocyte reaction occurred by reducing lymphocytes, migrating young monocytes and neutrophils into circulating blood, and *phagocytosis* of *bacterial* pathogens.

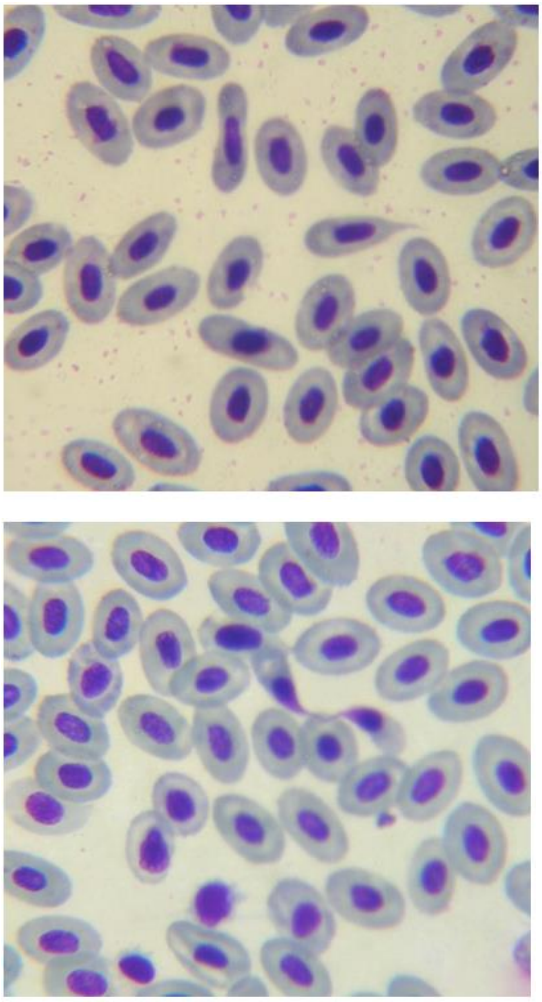

Figure 1. Rainbow trout blood smear, panoptic stain. Blood with signs of infection, qualitative changes in erythrocytes: poikilocytosis, schizocytosis [7]

Therefore, blood analysis provides relatively simple and fast tools for the assessment of fish health status.

Hematological parameters (number of erythrocytes, hemoglobin, hematocrit and their derivatives, total and differential white blood cell) can be of great importance for fish farmers serving as indicators of the fish physiological status, helping in the prevention and control of pathologies related to stress as a result of changes in the environmental components or to the action of pathogens.

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