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THE EFFECT OF AFLATOXIN FOOD CONTAMINATION ON THE IMMUNE RESPONSE (LEUKOCYTE REACTION) OF THE EUROPEAN CATFISH (*SILURUS GLANIS,* L. 1758*)*

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Abstract

Silurus glanis are an important romanian aquaculture species raised for food and sport fishing, especially in polyculture tehnology but also in the intensive semi-closed and recirculation sistems. The aim of present study was to obtain a basic knowledge of the leukocyte reaction of European catfish under the action of toxic metabolites (aflatoxin B) secreted by the mold Aspergillus flavus. The sampling of blood from the healthy and affected Silurus glanis exemplars allowed determination of white blood cell count. In order to achieve the purpose of the experiment the blood samples were immediately used to make smears which were colored with May-Gründwald Giemsa panoptic method. By studying different types of leucocytes was determined leukograma and absolute number of leukocytes. Physiological stress induced by toxic metabolites secreted by the mold is reflected in the hematological parameters - white blood cell count (significant decrease, p=<0.05). In the infected fish with aflatoxin the total number of leukocytes has been registered statistically significant changes with lymphopenia, neutrocytosisand eosinophilia. The hematological changes of infected fish led to the affect the immune defense system of the Silurus glanis specie.

Keywords: *aflatoxin B, european catfish, immundefence, white blood cell count (WBCc).*

INTRODUCTION

European catfish is an economically important cultured species in the Romanian aquaculture, reared especially in polyculture technology with cyprinids in the systematic and semi-systematic farms, began to be reared lately in the intensive, semi-closed and recirculating production systems (Docan et al., 2011). In fish culture, nutrition obviously plays an important role in the maintenance of a healthy and marketable product. Most of the diseases of nutritional nature represent the consequence of nutrition errors, lack of balances or deficiencies in the fodders composition, lent also of their contamination with toxic substances (Munteanu and Bogatu, 2003). The knowledge of the hematological characteristics is an important tol that can be used as an effective and sensitive index to monitos physiological a pathological changes in fish species (Kori-Siakpere et al., 2005). The differential leukocyte count has been used as an biomarker of physiological stress in a number of teleosts fish (Ellsaesser and Clem, 1986; Khangarot et al., 1999). Interpretation of changes in leukocytes proportions must be carefully considered when dealing with relative and absolute ratios. The analysis of leukocyte distribution within the peripheral blood of a fish may provide valuable insight into the function of the immune system in response to stress conditions (Ainsworth et al., 1991). The leukocytic reactions of the blood may be correlated with the pathogeny/pathogenesis of various acute or chronic, infectious, parasitical or toxic diseases. The importance of the hematological examination in the fish diseases diagnosis and in the mycotoxins effect evaluation has been widely accepted. The aflatoxins are mycotoxins with hepatotoxic and carcinogenic action, metabolism products of the *Aspergillus flavus* and *A. parasiticus* stems (Carlson et al., 2001). Therefore, even in the cases when the exposure to small dosage of aflatoxin does not lead to mortality, this is responsible for a higher sensibility to infectious diseases due to the insufficiency of the immune function (Sahoo and Mukherjee, 2001).

The aim of the present study was to obtain a basic knowledge of the immune response (measured changes in the distribution of leukocytes in the peripheral blood) of European catfish under the action of toxic metabolites

(aflatoxin B) secreted by the mold *A. flavus*, but also the assessment of the physiological changes.

MATERIALS AND METHODS

Fish biomass and the growing conditions. Fish biomass used in this study was represented by *Silurus glanis* specimens raised into a flow-through system of the pilot aquaculture station from the Aquaculture, Environment Science and Cadastre Department.

Fish were examined for any sign of infection or disease condition and only those fishes considered to be healthy were used for the study. The two experimental fish groups had individual mean weights of 682 ± 64.49 g/ex. in the first tank (C1), respectively 743 ± 41.79 g/ex. in second tank (C2). The stocking density was 74.7 kg/m³ for C1, respectively 74.3 kg/m³ for C2. The fishes from C1 were fed with fodder with 46% protein, and those from C2 with fodder with 30% protein. For both experimental types, the settled ration was of 1% BW per day. The fodder that was given to the fish from C1 was contaminated with *Aspergilus flavus*.

Blood sampling and analysis. The blood was sampled from 10 fish of each tank by caudal venous puncture using lithium heparin as anticoagulant, at the healthy and for the infected catfish. Blood was analysed with routin methods used in fish haematology (Blaxhall and Daisley, 1973). For each exemplar two blood smears were immediately dried, fixed and then colored with May-Grünwald Giemsa panoptic method (MGG). The relative proportion (percentage) of each type of white blood cells was obtained by microscopic examination of 200 leukocytes on blood smears. The type of leukocytes were determined based on identification characters listed by Svobodova (Svobodova et al., 1991). Absolute number of circulating blood leukocytes and thrombocytes were determined in relation to 1000 erythrocytes in haemograms stained with panoptic method MGG and converted to unit blood volume.

Statistical analysis. The different types of white blood cells (expressed as a percentage and absolute number) of the two experimental groups were expressed by mean and standard deviation and differences between the values were statistic analyzed with t-Student test.

RESULTS AND DISCUSSIONS

Following this experiment were also analyzed the reactions of the leukocyte's system, in order to determine the effect of the influence of aflatoxin in feed on the immune system defenses and for a fair assessment of physiological changes in *Silurus glanis*. To assess the leukocytemic changes were performed both qualitative analysis by observing the morphological particularities of the leukocytes and quantitative analysis to evaluate the relative changes (leukogram) and absolute changes (cells/µl blood) of different types of leukocytes. Microscopic examination of blood smears colored by MGG, did not show morphologic changes in leukocytes.

The results obtained after examining the blood smears are capable of supplying important information about the physiological state of the fish. In order to establish the effect of mycotoxin has upon the European Catfish's immune defence system, special attention was paid to the study of the leukocytic response.

The relative (the leukogram) and the absolute modifications of the various types of cells that make the leukocytic complex are given in Table 1.

Table 1. The relative and absolute modification of healthy and infected catfish

WBCc	SI units	Healthy catfish	Infected catfish
Leukocytes	$x10^3 \mu l^{-1}$	95.89±26.34	*42.46±8.08*^{a}
Lymphocytes	$x10^3 \mu l^{-1}$	76.67±27.88	*22.35±8.17*^{a}
	%	78.53±7.14	*51.48±9,12*^{a}
Monocytes	$x10^3 \mu l^{-1}$	2.89±1.08	*0.90±0.52*^{b}
	%	3.18±1.32	*2.21±1.3*^{b}
Neutrophiles	$x10^3 \mu l^{-1}$	16.10±5.49	*26.40±2.72*^{a}
	%	18.00±6.48	*45.15±8.28*^{a}
Eosinophiles	$x10^3 \mu l^{-1}$	0.24±0.05	*0.48±0.13*^{a}
	%	0.28±0.09	*1.17±1.11*^{a}

The morphological study of the blood smears both for the healthy catfish and for the infected one, clearly show a predominance of the lymphocytes, followed by the neutrophils (promyelocytes, metamyelocytesand neutrophils with kernel unsegmented or with

two or four lobs), monocytes and eosinophils (Figure 1). Basophiles were not found.

The analysis of the variation of the absolute number of ***leukocytes*** shows that these are severely reduced in the circulating blood of the catfish affected by mycotoxin. Thus, if in the case of the catfish in good physiological status, the blood smears presented approximately 95.89 $x10^3$ μl^{-1}, in the case of the diseased catfish, leucopoenia (a decrease of the number of leukocytes) was noticed. The leukocytes are significantly reduced by 55.7 % going down to 42.46 $x10^3$ μl^{-1}.

For fish, as for the other higher vertebrates, a general decrease in the number of leukocytes, as a consequence of repressing the natural defence immune system, is considered to be the result of acute stress (Ellis, 1977).

In order to emphasise the stressing effect of mycotoxin upon the physiological state of the catfish as well as its modus operandi we had to establish the leukocytic line responsible for the variation of the total number of leukocytes.

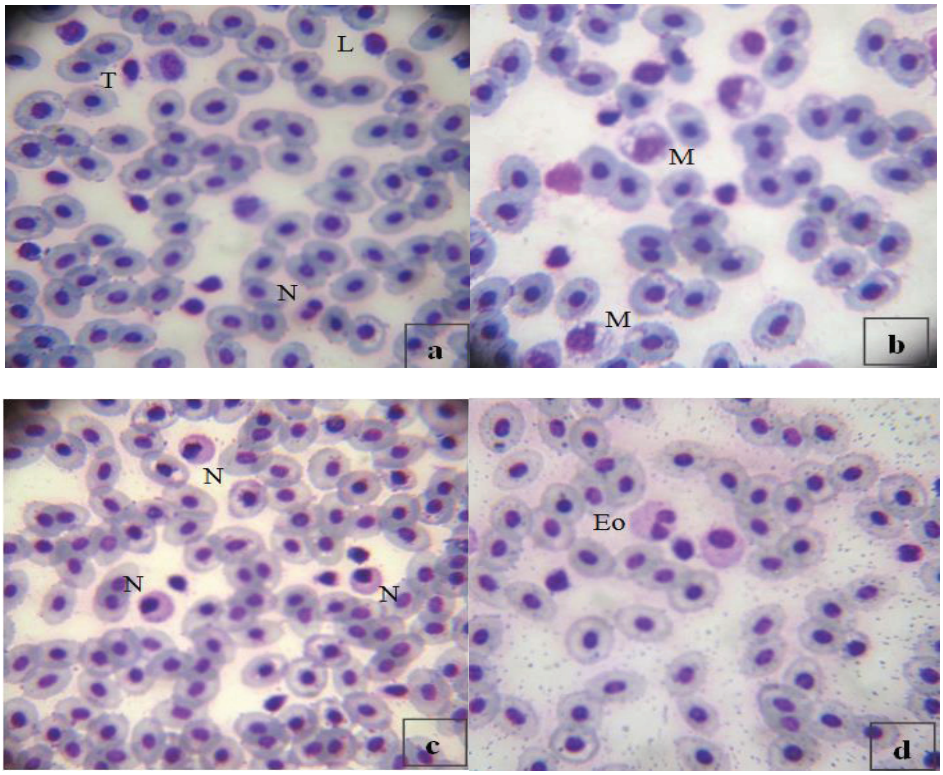

Figure 1. Light microscopic micrographs of peripheral blood of *Silurus glanis*:
a) L - lymphocyte, N - neutrophile;
b) M - monocytes with vacuolated cytoplasm;
c) N - young neutrophil, T - thrombocyte; d) Eo–eosinophile.

The monocytic reaction of the blood of the catfish affected by aflatoxicos is is different from the one of the healthy catfish. The number of the monocytes in the blood of the infected catfish decreases by 32.5 % as compared to the healthy catfish. As far as the absolute number of monocytes is concerned, we also notice a decrease by 69 % as compared to the healthy catfish. The monocytes transit only for 1-2 days through circulation, after which they reach the extravascular tissues and continue their maturation becoming functional as macrophages (Bârză, 1985).

The macrophages regulate the intensity of the antigenic stimulus but also interfere with the activity of the lymphocytes (Patriche, 2008). The study of the blood smears shows that, as compared to the total number of granulocytic

leukocytes, the neutrophils are the most numerous ones.

The neutrophilic reaction in the case of the catfish affected by aflatoxicosis is different from that of the healthy catfish.

We notice the statistically significant increase of the relative number of neutrophils by 150% as compared to the healthy catfish (from 18 to 45.15 %). The same ascending trend is preserved in the case of the absolute number of neutrophils as well (no. cel/µl blood) but only with 68 %.

In general, stress in fish induces granulocytosis (Ellis, 1977). Moreover, in the case of the species *Ictalurus punctatus*, a neutrophils level higher than 4% represents an indicator of the disease stress (Ellsaesser and Clem, 1986), and the relative neutrophilia is typical of the stress induced by manipulation, transportation, the percentage of neutrophils increasing from 4.2 ±1.5 to 20.9 ±1.5 %.

The microscopic examination of the blood smears in the catfish affected by aflatoxicosis showed the following: the increase of the absolute number of neutrophils is accompanied by the appearance in the blood flow of an increased number of myelocytes, metamyelocytes and young neutrophils. This increase in the number of young neutrophils characterises the initial stage of neutrophil fight. By means of phagocytosis, their main function, the neutrophils can neutralize bacteria, toxins or other alien substances in the organism (Bârză, 1985), leading to an increase in their number.

Eosinophilic granulocytes are generally characterised by motility and phagocytosis and have been correlated with the immune defence as well. The number of eosinophils in the blood of the catfish affected by aflatoxin was significantly higher, increasing approximately three times, counting for 1.17%. Expressed in absolute number of cells, eosinophils preserve the same ascending tendency reaching 0.48 $x10^3$ μl^{-1} in the infected catfish, respectively 2 times more as compared to the healthy catfish, which had only 0.24 $x10^3 \mu l^{-1}$.

Characteristic for eosinophilic granulocytes is the presence within the cytoplasm of certain secretory vacuoles whose product has the property of neutralising the toxic substances produced by some parasites. For these reasons the number of eosinophils may have increased in the blood of the catfish affected by aflatoxin precisely with the purpose of neutralising the toxin secreted by the *Aspergillius* strain. The increase in the absolute number of the circulating eosinophils suggests, more often than not from a pathogenic perspective, a reaction of late sensitivity (Bârză, 1985).

The answer of the ***thrombocytary system****,* under the stressful action of mycotoxin, is prompt and intense, manifesting through a thrombocytosis phenomenon (increase in the number of thrombocytes) in the circulating blood of the infected catfish. Thus, the absolute number of thrombocytes found in the blood smears increased, statistically significant ($p=0.002<0.05$) reaching 15.67 x 10^3 μl^{-1} in the case of the infected catfish, 112% higher than that in the blood of the healthy specimens, where the number of the thrombocytes did not exceed 7.38 $x10^3$ μl^{-1}.

The biotic factors (age, season, sex), the abiotic ones (water temperature, pH, content in the dissolved oxygen) as well as the stress may induce modifications in the number of thrombocytes (Tavares-Dias and Oliveira, 2009). The haemostatic mechanisms have been activated according to the stressing agent and its action, and this led to a rapid decline of the blood's coagulation time accompanied by a corresponding increase of the circulating thrombocytes in the blood. Practically, due to the increase of the corticosteroid hormones (catecholamine, cortisol) level that appears during stress the number of blood thrombocytes increases and the coagulation time decreases.

Results from this study support findings by others authors, which demonstrated that the immune response in fish can by affected by mycotoxins. The exposure of the rainbow trout fry to the action of B1 aflatoxin proved that this can generate long-term dysfunctions of the cellular immunoregulation, inducing a significant reduction of memory B cells (cells responsible for mediated humoral immunity, with rapid activation and long life). Moreover, we noticed the deletion of the immunoglobulin production but also of the proliferation of lymphocytes (Ottinger and Kaattari, 2000). In the case of Nile tilapia the toxin also affected other immunologic parameters with influence upon the degree of immunosuppression,

manifested through a reduction of the phagocytic activity of the macrophages.
Generally, in fish, the hepatoxic effect of the B1 aflatoxin generates the reduction of the total quantity of proteins due to an inhibition of the protein synthesis induced by the aflatoxin's binding to the cellular macromolecules, thus leading to the alteration of the production of humoral factors. On the other hand, the toxicity of the hematopoietic organs (the anterior kidney and the spleen) generate lymphocytolysis (attributed to the deterioration of the kidney tissue) as well as the reduction of the immunoglobulin production (Sahoo and Mukherjee, 2001). In fish, under the action of aflatoxin, a reduction of the agglutination bacterial titer along with an increase in the number of bacteria was noticed, which indicates in fact that chronic exposure to aflatoxin leads to the inhibition of the antimicrobial factors release (lysozyme and antiprotease) thus contributing to an increased susceptibility of fish to infectious diseases.

CONCLUSIONS

Since there are no real means to fight the negative effects produced by mycotoxins, the prevention of the fungal invasion by prophylactic measures is a necessity. Due to their resistance to heat, humidity and steam, it is recommended to check the ingredients necessary for the production of extruded feeds, so as to detect the presence of mycotoxins.
In the case of the catfish, the leukocytic and thrombocytic lines were the most affected ones, whose modifications materialised in a leukopenia and an accentuated 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, in the blood flow of the catfish affected by aflatoxin, by an increased number of young neutrophils, which proves a "regenerative reaction" specific to the initial stage of neutrophilic fight. The extremely active nature of this type of leukocytes as well as their intense phagocytic capacity are well known. The stressful effect of mycotoxin (produced by the metabolism of the strains of *Aspergillus flavus)* was felt by the catfish population leading to weakened and disordered metabolic processes by affecting the immunologic response cellular reaction which becomes obvious by the reduction in the number lymphocytes.
Lymphocytopenia accompanying the stress determined the reduction of the immunologic reactivity, leading to the appearance of certain agonal states which denotes a severe prognostic. Finally, the mortality among the biomass affected by aflatoxin was accentuated, reaching 83%.

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