Assessment of the Effect of Temperature on the Carp Physiology (*Cyprinus carpio*, L., 1758) Fed with Probiotics in Condition of a Recirculating Aquaculture System

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Abstract

In order to establish the effect of temperature on the physiology of the carp, special attention was awarded to leukograma and absolute number of leukocytes of carp blood. Body's reaction to stress factors action involves physiological changes, including changes in blood composition and immune mechanisms. The haematological analysis has been effectuated in the experiment that took place during 60 days, in four breeding units of 500 litters in volume each. Four kinds of variants were compared: V1-pellets with 30% crude protein, without probiotics; V2-pellets with 30% crude protein, with probiotics of 2.24×10^9 CFU/kg food; V3-pellets with 30% crude protein, with probiotics of 3.84×10^9 CFU/kg food; and V4-pellets with 30% crude protein, with probiotics of 7.04×10^9 CFU/kg food. We used BioPlus®2B probiotics (a mixture of *Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750). The microscopic examination of blood smears, which were coloured with May-Grunewald Giemsa panoptic method (both at the beginning and at the end of each experimental stage), we found that lymphocytes were predominant in comparison with other types of leukocytes, the absolute number being: V1 73.3–85.9%; V2 65.3–84.7%; V3 67.0–87.5%; V4 71.3–88.3%. It was noticed that eosinophilic granulocytes and basophils from the fish blood, were presented in a low number (0.1–0.8%), (0.2–0.4%) respectively, also at the beginning of the experiment and in the end of the two phases. The glucose concentration in blood registered higher values, across to normal values reported by literature for carp, also in control group (V1–101.8 mg/dl) as well as variants with different concentrations of probiotic (V2–104.6 mg/dl, V3–102.2 mg/dl, V4–116.2 mg/dl). The mean values of protein level were in the normal limits for carp, between 3.9 g/dl and 4.9 g/dl, aspect which suggests that fish present a normal physiological condition. In conclusion, probiotics can be used for enhancing the immune system and fish health, improving disease resistance of biological material.

Keywords: carp, leukocyte reaction, probiotics, RAS

1. Introduction

In order to determine the effect of temperature on fish physiology, studies regarding leukocyte array of carp species were performed. The organism reaction to stress factors involves physiological changes, including changes in blood composition and also, the ones related with mechanisms by which immune system responds [1].

The investigation of certain qualitative and quantitative characteristics of blood can lead to valuable information regarding the physiological state of culture biomass. Changes of certain blood characteristics represent the eco-physiological

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response of blood, fact that assures the organism survival in different environmental conditions [2].
Because there is a continuous dynamic equilibrium reported with the environment parameters, fish are very sensitive to physical-chemical changes and their defense reactions and mechanisms are clearly reflected by blood parameters [3].
Immunoglobulins IgM (antibodies) are proteins, mostly found in serum, involved in body defense mechanism against a series of pathogens as bacteria, viruses, fungi, vegetable or animal parasites. They are seen as particular elements of humoral immune response and also, they are synthesized by the organism as a specific defense response in case of a pathogen or foreign macromolecular compound penetration. The study of fish immunoglobulins represents the basis for many investigations which involves the phylogeny and ontogeny of their immune system and immune response characteristics.
The aim of this paper is to establish the effect of temperature on the physiology of the carp, special attention being awarded to leukograma and absolute number of leukocytes of carp blood.

2. Materials and methods

Regarding the leukocyte formula, blood smears were analyzed both at biological material stocking and harvesting moments, but also from the mid-term of current experimental period, in order to observe the changes that have appeared and that permit us to assess the influence degree of main technological factors. By studying the special scientific literature, it can be concluded that temperature variations induces certain effects on fish. In the present research, temperature was kept at a constant value during the whole experiment, the range value being between 13.5 and 16.8ºC, while the value of dissolved oxygen (DO) were in the range 4.2-8.1 mg/l.
The pilot recirculating grow system used for these experiments has been described, constructively speaking, also in other studies [4] reason for which this paper no longer contains its description. The production system mainly consists in 4 rearing units, with a volume of 1 m3 each and also water quality conditioning units.
The experiment was conducted over a period of 60 days, between December 2010–January 2011. The biological material used for this experiment was represented by carp juvenile, fish biomass being equally distributed into the four rearing units (V1-control and three different concentrations of probiotics: V2-2.24×10^9 CFU/kg food, V3-3.84×10^9 CFU/kg food, V4- 7.04×10^9 CFU/kg food).
The relative and absolute number of leucocyte was obtained by microscopic examination of 200 leukocytes on blood smears (two per each fish), using *Zeizz Axio Imager* microscope and immersion objective (10 oc. X 100 ob.). Absolute number of circulating blood leukocytes and thrombocytes was determined in comparison with 1000 erythrocytes counted on hemocytometer, per blood volume unit. The blood smears were colored with *May-Grünwald Giemsa* panoptic method (MGG) and the type of leukocytes were determined based on identification characters listed by [5].
The determination of serum biochemical parameters (glucose, total protein, immunoglobulins) was performed in both the laboratory of Medicine and Pharmacy Faculty, "Dunărea de Jos" University of Galaţi and laboratory of "St. John" Children Emergency Hospital, Galaţi. After separating the serum by using centrifugation (10 minutes at 3000 rpm), serum samples were analyzed by using dry biochemical auto-analyzer *VITROS 750* and *IMOLA chemistry analyzer* for wet biochemistry, after human standardized methods, modified for fish. In order to reduce the influence of diet on biochemical parameters values, fish were not fed the day before sampling.
The sampling for the biochemical analysis was performed in three important stages: at biological material stocking and harvesting moments and also, from the mid-term of current experimental period. For this blood analysis, a total number of 60 blood samples were collected (20 blood samples for each stage of those mentioned above) from fish that were reared in recirculating aquaculture system conditions, under different technological regimes, by administrating different concentrations of probiotics in their daily feeding ratio.

3. Results and discussion

After the microscopic examination of blood smears, MGG stained, lymphocytes were dominated, compared with other types of leukocytes, being present in a large number, fact valid for carp in all four experimental variants (both at the beginning and end of each stage). It can be noticed a very low presence of eosinophilic and basophils granulocytes in carp blood stream, both at the beginning and at the end of each experimental stage.

By analyzing of certain different types of leukocytes and also, by determining the mean percentage value of leukocytes, it can be observed a reduction in lymphocytes, while monocytes and neutrophils percentage increased, after administrating probiotics *Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750) in feed. The presence of eosinophils and basophils granulocytes in blood was not significant.

Through their enzymatic equipment and also, their content of vasoactive substances, leukocytes (histamine, serotonin) interfere within the general systemic reactions. Leukopenia (the process of decreasing the number of leukocytes) is a hematological parameter recognized as a secondary indicator of stress [6, 7].

The cytological examination performed on blood smears have revealed a series of qualitative changes for erythrocytes, consisting in the appearance of cells having different shapes, sizes or being in under-going division (Figure 1) [8].

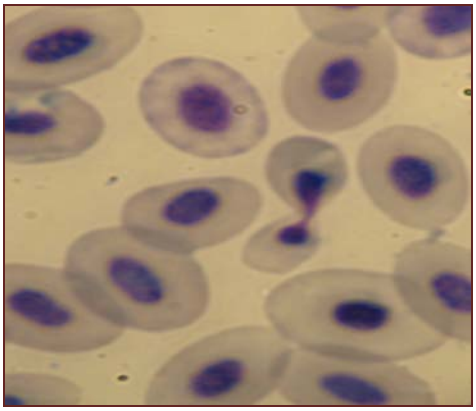

Figure 1. Erythrocytes in division

Where red blood cells into under-going division are observed in blood, it means that they are adaptive changes that facilitate the production of large amounts of energy [9, 10].

The obtained results reveals differences between the lymphocytes level at the end of the first experimental stage, values which are lower compared with the ones from the beginning of the experiment. Also, it must be pointed that the lymphocytes number has an upward tendency towards the end of the last experimental stage (Figure 2).

Similar results were obtained by other author as [11], who demonstrated that thermal shock generates a decrease in the number of lymphocytes-B and increases granulocytes for *Cyprinus carpio*, respectively *Salmo salar*.

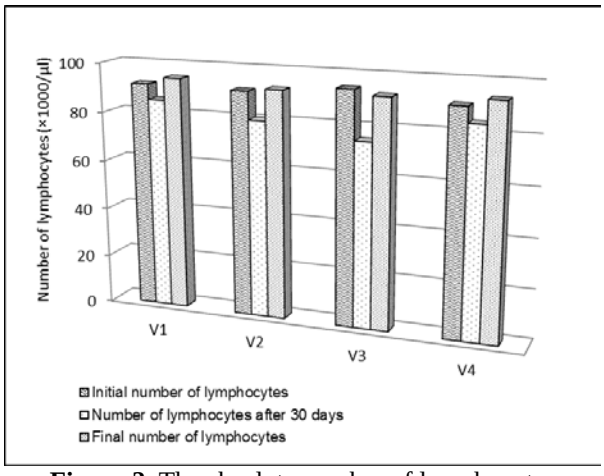

Figure 2. The absolute number of lymphocytes (Initial, after 30 days and final of the experiment)

By analyzing figure 3, we can see that the absolute number of monocytes mean value indicates significant differences between the beginning and the end of the first experimental stage, but also between the experimental variants, as follows: in V3 initial variant, the absolute number of monocytes was 2.5×10^3 cel/µl and it increased to 12.7×10^3 cel/µl at the end of the first stage and then decreased to 2.9×10^3 cel/µl at the end of last experimental stage period; the other variants revealed the same trend, a more accentuated upward tendency being valid only for control version.

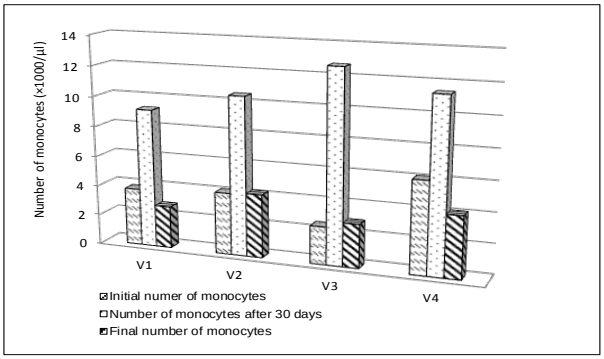

Figure 3. The absolute number of monocytes (Initial, after 30 days and final of the experiment)

At the end of both experimental stages, the microscopic examination revealed the presence of blood segmented and ribbon neutrophils and also, metamyelocytes, whose cytoplasm is pale colored and has a very fine granulation. As regarding the neutrophil granulocytes, a major increase can be observed at the end of first experimental stage, in particular for V3 experimental variant, where probiotic average concentration was administrated, compared to the control (V1). This thing was followed by a significant reduction of those elements, at the end of the experimental period (Figure 4).

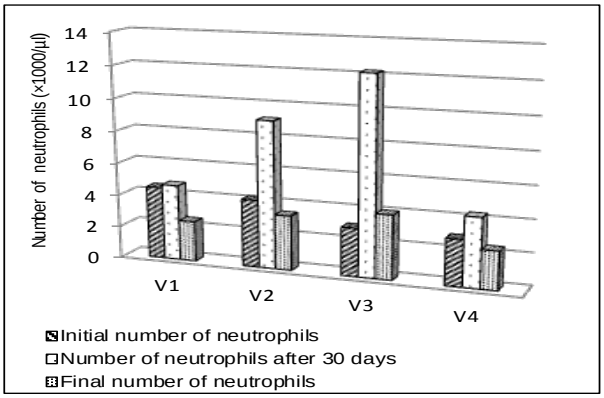

Figure 4. The absolute number of neutrophils (Initial, after 30 days and final of the experiment)

Eosinophils and basophils granulocytes were present in a low number in blood, segmented lobe eosinophils and metamyelocytes being the forms encountered, while basophilic granulocytes were found only in carp specimens, at the end of the second experimental stage, in all variants except V4.

Thrombocytes are cells of the immune system, produced by spleen, involved in nonspecific defense mechanisms, acting as a true protection barriers. A change of their number may reflect a certain physiological state of the organism [12]. It was found that the thrombocytes number was lower at the end of the first experimental stage, reported with the initial moment, fact valid for all the experimental variants except V2, where an increasing number of thrombocytes had been registered, water temperature recording values close to optimal range from the technological point of view (Figure 5).

According to some authors, such as [13], in their complex papers regarding blood coagulation system in fish, it can be noticed that a series of biotic (age, season, sex) and abiotic factors (water temperature, pH, dissolved oxygen concentration) and also stress, can induce changes in the number of thrombocytes. Depending on the stress agent and its action, there was observed an activation of homeostatic mechanisms, fact that induced a rapid decline in blood clotting time, in accordance with an increase of the number of blood thrombocytes.

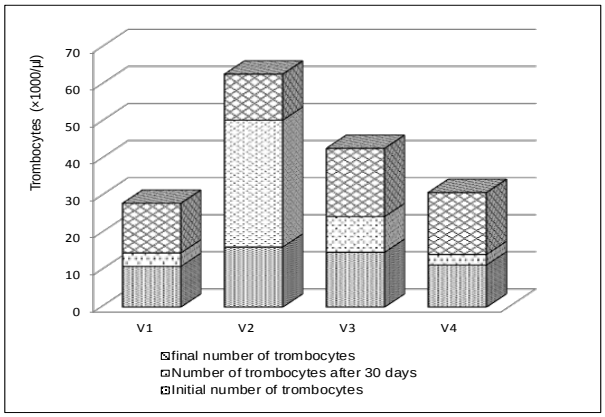

Figure 5. Number of Thrombocytes (Initial, after 30 days and final of the experiment)

In the experiment conducted by [14], regarding the influence of probiotic *Bacillus subtilis* and *Lactobacillus acidophillus* on the natural immune response and the resistance of Nile tilapia (*Oreochromis niloticus)* at the occurrence of infections, it is suggested that probiotics may enhance the appearance of nonspecific immune responses. Fish to whom a mixed of *Bacillus subtilis* and *Lactobacillus acidophillus* was administrated, had a higher survival rate, comparing with infected fish that have not received the above mentioned probiotic mixture. Similar results have been reported in the literature, where an increased survival rate was observed among channel catfish (*Ictalurus punctatus*)affected by parasites and feed with

pellets containing *Bacillus spp*. and *Bacillus S11* [15].

The blood biochemical analysis

For characterizing the nutritional and maintenance status of the biological material in the growth experiment where different concentrations of probiotic were administrated, blood biochemical analyses were done.

The results obtained after the determination of the biochemical parameters in serum are presented in table 1, 2, and 3.

An increase of glucose level above the normality range was found in all chronic or acute states of stress, but also in the early stages of infectious diseases. Generally, aquaculture industrial activity involves practicing high stocking densities, fact that decreases the space corresponding to each specimen. The direct consequences of those that were mentioned are manifested through the emergence of neurohormonal changes and also of stress, facts that directly imply the concentration of glucose in blood.

Table 1. The values recorded at the beginning of the first phase for serum biochemical parameters

Experimental variants	Glucose (mg/dl)	Total proteins (g/dl)	Immunoglobulin (mg/dl)
	X±SD	X±SD	X±SD
V1	101.8±42.00	4.64±0.26	38.7±2.64
V2	104.6±18.10	4.12±0.14	40.00±2.05
V3	102.2±53.65	4.4±0.3	40.14±2.44
V4	116.2±20.31	4.26±0.27	39.13±1.72

Table 2. The values recorded at the end of the first experimental phase (beginning of the second stage) for serum biochemical parameters

Experimental variants	Glucose (mg/dl)	Total proteins (g/dl)	Immunoglobulins (mg/dl)
	X±SD	X±SD	X±SD
V1	71±33.06	4.9±0.18	38.83±1.38
V2	95.8±19.26	4.4±0.15	40.74±3.57
V3	118±44.34	4.48±0.54	40.6±5.93
V4	115.8±11.88	4.08±0.14	37.27±1.67

Table 3. The values recorded at the end of the second stage for glucose and total serum protein

Experimental variants	Glucose (mg/dl)	Total proteins (g/dl)
	X±SD	X±SD
V1	76.8±5.54	4.2±0.30
V2	99±24.01	4.02±0.32
V3	98.4±10.06	3.9±0.18
V4	118.8±19.79	4.04±0.45

To keep the glucose within certain normal limits is one of the mechanisms with the finest homeostatic adjustment, to which the hepatopancreas participates, as well as some extrahepatic tissues and a series of endocrine glands [16-21].

Blood hyperglycemia is the most commonly used indicator for assessing the variation that occurs during the response time of fish to stress conditions [22]. Other studies [23] have demonstrated that glucose in the blood increases, as a normal response to the appearance of fish stress, but the extent and duration for maintaining it at a higher level proved to be strongly dependent on fish species and nutritional status.

Differences were recorded for carp reared in the mentioned recirculating aquaculture system, in terms of blood glucose concentrations, determined in each of the three sampling moments. Thus, at the beginning of the experimental period, blood glucose levels had registered high values, over the normality range upper limit, in case of control variant (V1), as well as for variants where different concentrations of probiotic were administrated. At the end of the first stage, blood glucose presents an upward tendency in case of variants where different concentrations of probiotic were administrated compared to the control variant, where the concentration of serum

glucose is within the normality range. At the end of the experimental period, blood glucose values are higher at V4 experimental variant, compared to the control variant (V1).

Total serum proteins, related to variants where different concentrations of probiotics were administrated, shows a slight decrease compared to the control variant, both at the beginning and at the end of experimental period. Also, following the determinations that were made, the mean values of total serum protein were within the range of 3.9 g/dl and 4.9 g/dl, fact which reveals that fish has a normal physiological condition, given by this biochemical serum parameter and also, respecting the normal range for fish growth.

From the obtained results it can be stated that immunoglobulins presents differences between control variant V1 and V2, V3 variants, both at the beginning and at the end of first experimental stage. The average values immunoglobulins ranged between 38.7 mg/dl at the beginning of the experiment and 40.74 mg/dl at the end of first experimental stage. At the end of the experimental period, the presence of total serum immunoglobulin has not been detected, although analysis of samples had been made repeatedly. This can be explained by the presence of stress factors. Therefore, stress response is the main reason for the above statement. This response is generated by the technological conditions, described above, where temperature has the most significant effect.

Factors such as age, sex, environmental conditions and diet have a significant influence on serum biochemical values [23].

According to the literature [8], it is known that the temperature of the growth medium is a factor of stress that leads to the inhibition of potentially phagocytic cells, their activity being more reduced at a lower temperature. This implies that the functionality of the immune system is more efficient at higher temperatures, fact supported also by the higher immunoglobulin level. So, it results the inhibitory effect on the immune system generated by low growing temperatures, compared to a more marked secretion of humoral immune effectors, at high temperatures.

4. Conclusions

By analyzing the data regarding the hematological and biochemical indicators which reveals the changes that had appeared under the influence of main technological factors on the evaluation of physiological state of carp, reared in a recirculating system, it can be mentioned that probiotics can be used to strengthen the immune system and health status, thereby improving the biological material resistance to diseases and increasing its growth rate.

The level of blood glucose and total protein are easily modified under the influence of external or internal factors. This explains their importance, as biochemical reference indicators, in assessing the normality level of general physiological state, for the biological material used in present study.

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