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EFFECTS OF SHORT-TERM STARVATION AND DIFFERENT DIETARY PROTEIN LEVEL ON LEUKOCYTE REACTION IN CULTURED RAINBOW TROUT *Oncorhynchus mykiss* (Walbaum, 1792)

Mirela CREȚU, Raluca Cristina ANDREI (GURIENCU), Lorena DEDIU, Angelica DOCAN, Mihaela MOCANU

"Dunărea de Jos" University of Galați, 47 Domnească Street, 800008, Galați, Romania

Corresponding author email: lorena.dediu@ugal.ro

Abstract

Study of the physiological and haematological characteristics of cultured fish represents an important tool in monitoring environmental quality, physiological status and the health condition of fish. The purpose of this paper was to evaluate the leukocyte reaction of rainbow trout after applying short periods of starvation (2 days and 4 days) and re- feeding with different dietary protein levels. Six experimental variants in duplicate were created, as follows: two control groups, feed daily, ad libitum, with commercial pellets containing 41% crude protein (D41) and 50% crude protein (D50); two groups starved for 2 days (D2) and then fed with commercial pellets with 41% crude protein (D2/41), respectively 50% crude protein (D2/50) and two groups starved for 4 days (D4) and then fed with commercial pellets with 41% crude protein (D4/41), respectively 50% crude protein (D4/50). In order to determine the leukogram and absolute number of leukocytes, blood samples were taken, at the beginning and at the end of the experiment, in order to make smears that were coloured with May-Grunewald Giemsa panoptic method. Regarding the leukogram of rainbow trout it can be observed an insignificant decrease ($p>0.05$) of the relative number of lymphocytes, respectively an insignificant increase ($p>0.05$) of the relative number of neutrophils, while the case of the relative number monocytes, it was observed a significant decrease ($p<0.05$) in fish starved for 2 days. The absolute number of leukocytes ($\times 10^3$ cells/µl bloods) registered no statistically significant changes ($p>0.05$) between the experimental groups, while the absolute number of monocytes showed a significant decrease for the fish starved for 2 days. However, it can be concluded that application of short periods of starvation did not affect the immune defence system of the rainbow trout fish.

Key words*: leukocyte reaction, protein level, rainbow trout, starvation.*

INTRODUCTION

Both in natural conditions and in aquaculture, fish can experience periods of fasting (Barcellos et al., 2010; Rahmati et al., 2019). In aquaculture, starvation of fish could occur due to an inadequate diet or feeding protocols (López-Olmeda et al., 2012), or can be used as a feed management strategy to reduce the feeding cost (Blanquet & Oliva, 2010; Xiao et al., 2013) or to solve some water quality problems caused by overfeeding the fish (Turano et al., 2008).

According to FAO 2009, rainbow trout is a fast-growing fish species which have become highly economically important both globally and more specifically in Europe. Recently, the modern trout farming practices are targeted towards optimizing feed conversion by using high-energy, high-fat, low-protein diets, but such practices are not always acceptable to the consumers. In this context, starvation of fish is can also be practiced in order to hold back stocks to regulate supply in accordance with consumer demand and to reduce excess lipid levels.

There are many studies who demonstrated that after some period of starvation or exposure to unfavourable conditions such as low temperature, low oxygen, and reproductive effort, fish can experience an accelerated growth rate, a phenomenon called compensatory growth (Tian and Qin, 2003; Ali et al., 2003; Adakli and Tasbozan, 2015).

Generally, these starvation periods might induce some hormonal and biochemical changes at fish. The primary responses to stress can be described as the activation of the neuro-endocrine system through the release of stress-related hormones (cathecolamines and cortisol) in the blood, while secondary responses include hematological and biochemical changes.

Also, during starvation, the muscle glycogen is reduced, and fat or protein are mobilized aspect which can lead to impair fish meat quality (Barcellos et al., 2010; Sigholt et al., 1997; Thomas et al., 1999).

Blood parameters are commonly used as a suitable tool for clinical diagnosis, particularly in assessing the health and nutritional status of fish and can be used to evaluate the effect of starvation periods on fish welfare.

Information regarding the effects of feeding and starvation on haematological values of rainbow trout is available but there was no information about the effects of these factors on the hematopoietic system. There are some authors who say that even short periods of starvation might weaken the immunological system of fish (Caruso et al., 2010; Caruso et al., 2011; Shoemaker et al., 2003).

In this context, the aim of the present study was to evaluate the leukocyte reaction of rainbow trout after applying short periods of starvation (2 days and 4 days) and refeeding with different dietary protein levels (41% and 50%).

MATERIALS AND METHODS

Blood samples and analysis. Fish (initial weight 111.93±15.76 g; initial length 21.35±1.04 cm) were provided from a growth experiment, which lasted for 46 days. The experiment was carried out in the facility of the "Dunărea de Jos" University from Galați, Faculty of Food Science and Engineering, Romania.

Six treatments with duplicate were assigned, as follows: two control groups, feed daily, *ad libitum*, with commercial pellets containing 41% crude protein (D41) and 50% crude protein (D50); two groups starved for 2 days (D2) and then fed with commercial pellets with 41% crude protein (D2/41), respectively 50% crude protein (D2/50) and two groups starved for 4 days (D4) and then fed with commercial pellets with 41% crude protein (D4/41), respectively 50% crude protein (D4/50). The biochemical composition of the feed is presented in Table 1. At the end of the experiment, about 1 ml of blood was taken by caudal venous puncture, from 5 fish/on each experimental variant and blood smears were prepared and stained using the methods of Giemsa and Pappenheim (Wintrobe, 1967). The blood smears were used for microscopic observations. In total, we analyse 120 blood smears. The relative proportion of each type of white blood cells was obtained by microscopic examination of 200 leukocytes on blood smears. The absolute number of circulating blood leukocytes and thrombocytes were determinate in relation to 1000 erythrocytes and converted to unit blood volume.

Table 1. Biochemical composition of experimental diets

Ingredients	Composition	Diet41	Diet50
Crude protein	%	41	50
Crude fats	%	12	20
Crude fiber	%	3	0.7
Crude ash	%	6.5	8
Phosphorus	%	0.9	1.2
Vitamin A	UI	10000	6000
Vitamin D3	UI	1250	1200
Digestible energy	MJ/kg	14.2	19.7
Ingredients: Fish meal, fish oil, haemoglobin, full-fat soybean, soybean oil, wheat gluten, sunflower flour, wheat, and wheat products.			

Statistical analysis. Values obtained for the percentage and the absolute numbers of different types of white blood cells were presented as mean and standard deviation.

The obtained data were subjected to analysis of variance (ANOVA) to test the effect on the two factors. Duncan's multiple range test was used as a post hoc test to compare means at $p<0.05$. the SPSS version 21 software was used**.**

RESULTS AND DISCUSSIONS

The objective of this study was to examine welfare aspects, including the response to stress due to starvation (cycles of two days and four days) and refeeding with different dietary protein levels (41% and 50%).

Usually, in aquaculture fish are exposed to periods of starvation or restricted feed intakes. In these periods, the fish covers the energy requirements on the expense of body stores of nutrients, situation that can lead to the alteration of the physiological state in time (Lie and Huse, 1992).

Microscopic examination of blood smears coloured with MGG, did not show morphologic changes among leukocytes. Following microscopic examination of blood smears, stained by the MGG method, it was observed that lymphocytes dominated in comparison with the other types of leukocytes, being present in a very large number (Figures 1-3).

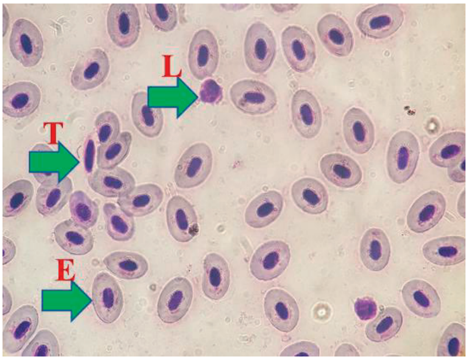

Figure 1. Morphology of circulating blood cell of the rainbow trout (L-lymphocytes, T-thrombocytes, E-Erythrocytes, 10 oc x 100 ob)

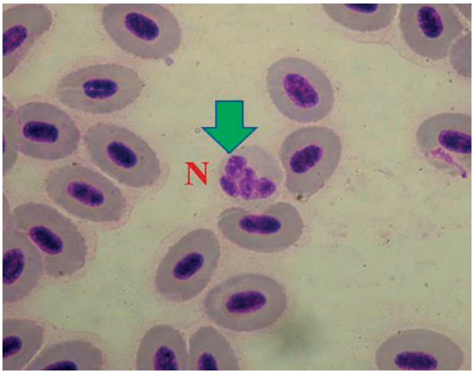

Figure 2. Morphology of circulating blood cell of the rainbow trout MGG staining (N-neutrophil, 10 oc x 100 ob)

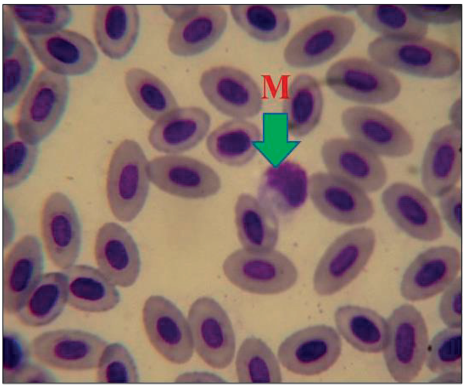

Figure 3. Morphology of circulating blood cell of the rainbow trout MGG staining (M- monocyte, 10 oc x 100 ob)

No eosinophils or basophils were found in fish from this study.

Compared with control groups (D41 and D50) the relative number of lymphocytes, showed an insignificant decrease ($p>0.05$) in variants starved for two and four days respectively. However, a decrease of these values was observed with the increase of the starvation period (Figure 4).

Regarding the average percentage value of monocytes, there is a non-uniform evolution. In fact, statistical analysis showed that the relative number monocytes registered a significant decrease ($p<0.05$) in fish starved for 2 days. Thus, if in the case of fish from control groups and those starved in 4-day cycles, a slightly higher value was observed (D41 - 0.67±0.12%; D50 - 0.50±0.14%; D4/41 - 0.83±0.16; D4/50 - 0.75±0.17%) for fish starved for 2-day cycles, the percentage of monocytes showed a significant decreases to 0.30% (D2/41 - 0.30± 0.11%; D2/50 - 0.30±0.13%) (Figure 4).

Regarding the average percentage of neutrophils those shows an insignificant increase ($p>0.05$) with the increase of starvation period (Figure 4).

Concerning the absolute number of cells reported (×10^3 cells/µl blood), it can be observed that they are dominant comparing to other types of leukocytes (Table 2).

The number lymphocytes, neutrophils, and monocytes are important indicators of fish health and one of the main parts of the body's non-specific immune system (Ahmadifar et al., 2009). In our study, ANOVA analysis showed no statistical ($p>0.05$) differences for the values of absolute number of leukocytes, lymphocytes, neutrophils or thrombocytes for the fish across all treatments.

The absolute number of leucocytes was not influenced ($p>0.05$) by the protein content of the administrated feed. However, lower values were observed in the case of 41% crude protein. Also, it can be observed that the absolute number of leucocytes registered an insignificant decrease with the increasing of the starvation period.

According to some authors, after applying of short stress period it can be observed a diminishing of the leukocyte count- leukopenia (Falcon et al., 2008). The authors explain this reaction is due to the release of corticosteroids and catecholamines. These hormones favour haemoconcentration, due to the increased interstitial pressure that promotes an increase in fluid passage to the interstitial space (Allen and Patterson, 1995). Thus, the leukocytes are redistributed from the blood vessels to the tissues, causing immunosuppression (Koser and Oliveira, 2011). Similar studies showed the reduction of the absolute number of leucocytes after short-term starvation in the case of *Huso huso* (Morshedi et al., 2011). Also, Rios et al. (2011) reported leukopenia (lymphocytopenia) and thrombocytopenia in starved *H. malabaricus*.

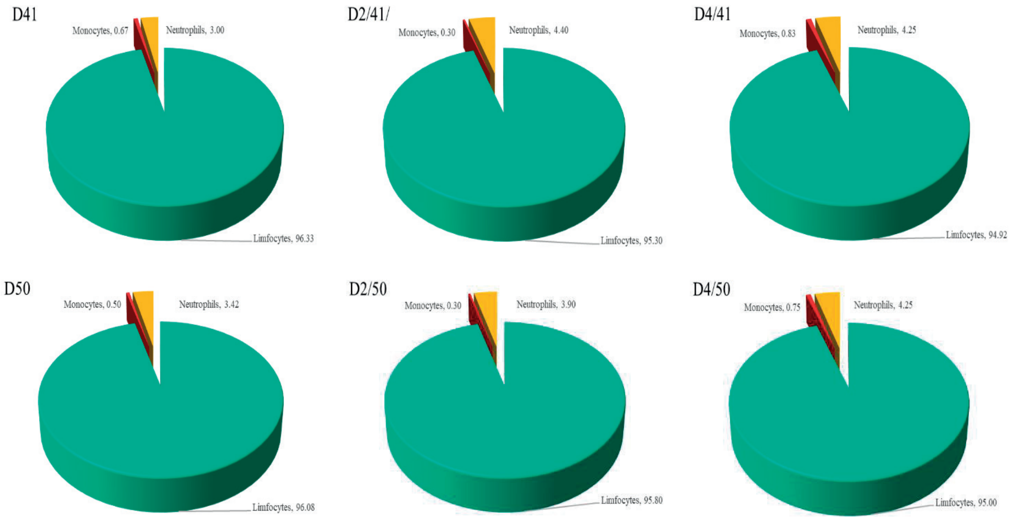

Figure 4. The leukogram (%) of rainbow trout at the experimental variants

Table 2. Changes in absolute values of the leucocyte series at the end of the experimental period

Experimental variants	Means ± SD (×10^3 cells/µl blood)				
	Leucocytes	Lymphocytes	Monocytes	Neutrophils	Thrombocytes
D_{41}	67.36±16.19	64.99±16.16	0.41±0.01	1.96±1.07	5.31±1.20
$D_{2/41}$	63.54±15.36	60.71±15.36	0.15±0.04	2.67±0.27	4.20±1.45
$D_{4/41}$	61.64±12.51	58.50±17.08	0.50±0.03	2.64±0.85	3.40±1.29
D_{50}	78.41±13.68	75.49±13.76	0.49±0.08	2.64±1.31	7.28±1.35
$D_{2/50}$	70.70±12.99	67.74±12.45	0.19±0.04	2.77±0.94	6.11±1.27
$D_{4/50}$	63.65±17.79	60.80±17.20	0.47±0.03	2.70±1.61	5.35±1.09

Also, the absolute number of lymphocytes at the end of the experimental period was not influenced by the protein content from the fed ($p>0.05$) or by the period of starvation. However, slightly lower values were observed in fish fed with 41% crude protein, as well as in fish starved in 2 and 4 days**.**

Fish monocytes are involved in a specific immunity as phagocytic cells and in the specific immune response as antigen-presenting and immunomodulating cells.

Regarding the absolute number of monocytes, it was observed significant decrease ($p<0.05$) in fish starved for 2 days (D2/41 - 0.15±0.04 ×10^3 cells/µl, D2/50 - 0.19±0.04×10^3 cells/µl), while in the case of fish starved in 4-day cycles, the absolute number of monocytes registered no significant differences ($p>0,05$) compared to the fish fed daily (D4/41 - 0.50 ± 0.03 cells/µl, D4/50 - 0.47 ± 0.03 cell/µl).

The content of crude protein from feed did not influence the absolute number of monocytes.

By comparing the absolute number of neutrophils released in the blood, statistically insignificant differences are observed ($p>0.05$), the number being higher in the case of the variant were fish were starved in comparation with the control groups. Slightly higher values were observed in fish fed with 50% PB feed, but no significant differences were recorded (p>0.05).

Fish thrombocytes are involved in blood clotting, phagocytosis, and other possible immunologic functions. In our study the absolute number of thrombocytes, did not reveal significant differences (p>0.05), but higher values were recorded in the variants were fish were fed with 50% crude protein.

Regarding the influence of starvation period on absolute number of thrombocytes it was observed a reduction with the increase of starvation period.

The physiological effects of starvation on fish welfare may vary considerably in relation to fish species and age, as well as to the length of the period of starvation. Although in our study, the leukocyte reaction was not significantly influenced by the starvation periods applied, further studies are needed to elucidate all the mechanisms involved.

CONCLUSIONS

The results of present study indicate that applying of short period of starvation on rainbow trout and refeeding with two different levels of protein (41% and 50%) induce no significant changes in terms of leukocyte reaction.

However, starvation caused a slight reduction of absolute number of leukocytes and lymphocytes, which is a response of fish organism caused by stress. These data show that innate immune mechanisms in fish are insensitive to dietary protein level but may be compromised if the starvation is applied for a longer period.

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