

## Effect of Stocking Density on Leukocyte Reaction of *Oncorhynchus mykiss* (Walbaum, 1792)

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### Abstract

This study evaluates the hematological response of rainbow trout fingerlings to crowding stress induced by high stocking densities, during a 33 days period. A number of 254 rainbow trout, with an average weight of  $29.51 \pm 1.32$  g, were divided into four rearing units in order to create different stocking densities: in DS<sub>1</sub>-2.64 kg/m<sup>3</sup>, DS<sub>2</sub>-5.16 kg/m<sup>3</sup>, DS<sub>3</sub>-7.12 kg/m<sup>3</sup> and in DS<sub>4</sub>-9.42 kg/m<sup>3</sup>. To determine the leukograma and also the absolute number of leukocytes, blood samples were collected after the experimental trial and used immediately to make smears. Physiological stress induced by maintenance under different stocking densities is reflected by rainbow trout leukograma. We can notice a decrease of absolute number of leukocytes, with direct implication of stocking density level, from  $69.79 \pm 10.22$  ( $\times 10^3$  cel./mm<sup>3</sup>) in DS<sub>1</sub> to  $46.34 \pm 9.83$  ( $\times 10^3$  cel./mm<sup>3</sup>) in DS<sub>4</sub>. In this experiment, the immune (defense) system has been influenced by stocking density, the general reduction of leukocytes number being a characteristic response to stressful conditions.

**Keywords** leukograma, rainbow trout, recirculating aquaculture system, stocking density

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### 1. Introduction

The apparent efficiency of aquaculture systems can be maximized by increasing the rearing densities. High stocking density conditions has been reported to decrease growth in salmonids due to different factors such as low feed consumption and social interactions or inappropriate water quality.

The effects of stocking density on the physiology and maintenance conditions of fish have been intensively researched by experts in aquaculture. When space requirements conditions are not corresponding with the normal ones, stress related to captivity occurs, producing a physiological specific response. Captivity stress is often associated with high stocking densities and it can lead to super high density stress (or congestion). Under the action of high stocking density stress,

fish undergo physiological changes, including changes in hematological parameters, which represent a quantification method to assess the response of fish in relation to stressful conditions. Red blood cells parameters can be used for the diagnosis of anemia while the leukocytes, the primary line of immunological defense, provide an important representation of defense cells throughout the body [1].

Thus, one of the most elementary ways to assess the immune system is to explore changes in the number or appearance of fish circulating white blood cells.

The objective of the present study is to evaluate the effects of stocking density on the hematological parameters of rainbow trout (*Oncorhynchus mykiss*), in order to determine the technological factors influence on their physiological state.

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## 2. Materials and methods

This experiment was carried out in the experimental recirculating system of the Aquaculture, Environmental Sciences and Cadastre Department, "Dunărea de Jos" University of Galati. This recirculating system described in our precedent papers [2], consist mainly in a living space represented by 4 rearing units of 0.320 m<sup>3</sup> each (40×80×100 cm) and the conditioning unit of the water quality. The biological material, 254 rainbow trout fingerlings (*O. mykiss*), with an average weight ( $\pm$ SD) of

29.51 $\pm$ 1.32 g, were divided into four rearing units in order to create different stocking densities. The initial distribution of individual weight values, for all variants, showed a significant similarity with the normal distribution ( $p > 0.05$  KS test). For this experiment, the feeding intensity was 3% of body weight (% BW) per day (for the first six days), respectively 4 % of body weight per day (for the following 27 days of the experiment). The pellets were manually distributed, two times per day. The feed used was Nutra PRO MP-T - extruded pellets with the diameter of 1.7 mm and 50% protein content.

**Table 1.** The synthetic table regarding the experimental version

Technological indicators	DS <sub>1</sub>	DS <sub>2</sub>	DS <sub>3</sub>	DS <sub>4</sub>
Number of fish	25	51	75	103
Initial biomass (g)	792	1550	2138.94	2828.20
Mean individual weight (g/fish)	31.68	30.39	28.52	27.46
Initial stocking density (kg/m <sup>3</sup> )	2.64	5.16	7.12	9.42
Final numbers of fish	25	50	75	103

At the beginning and at the end of the experiment, biological samples of blood (0.3-0.5 ml) were collected from 10 fish, by puncture of the caudal vein using lithium heparin as an anticoagulant. Knowing the fact that 2-phenoxyethanol anesthesia had no effect on hematological profile [3], fish were anesthetized with 2-phenoxyethanol in order to reduce handling stress.

For each fish, two blood smears were immediately dried, fixed with methanol and then colored with May-Grünwald panoptic method (MGG). The relative proportion 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, Z. [4]. Absolute number of circulating blood leukocytes and thrombocytes was determined in comparison with 1000 erythrocytes counted on haemocytometer, per blood volume unit. The statistical analyses were made in Microsoft Excel and SPSS 17( K-S test). For leukocytes (expressed as a percentage and absolute number), mean values and standard deviation were calculated.

## 3. Results and discussion

The research presented in this paper is part of a larger study that assessed the normality degree of

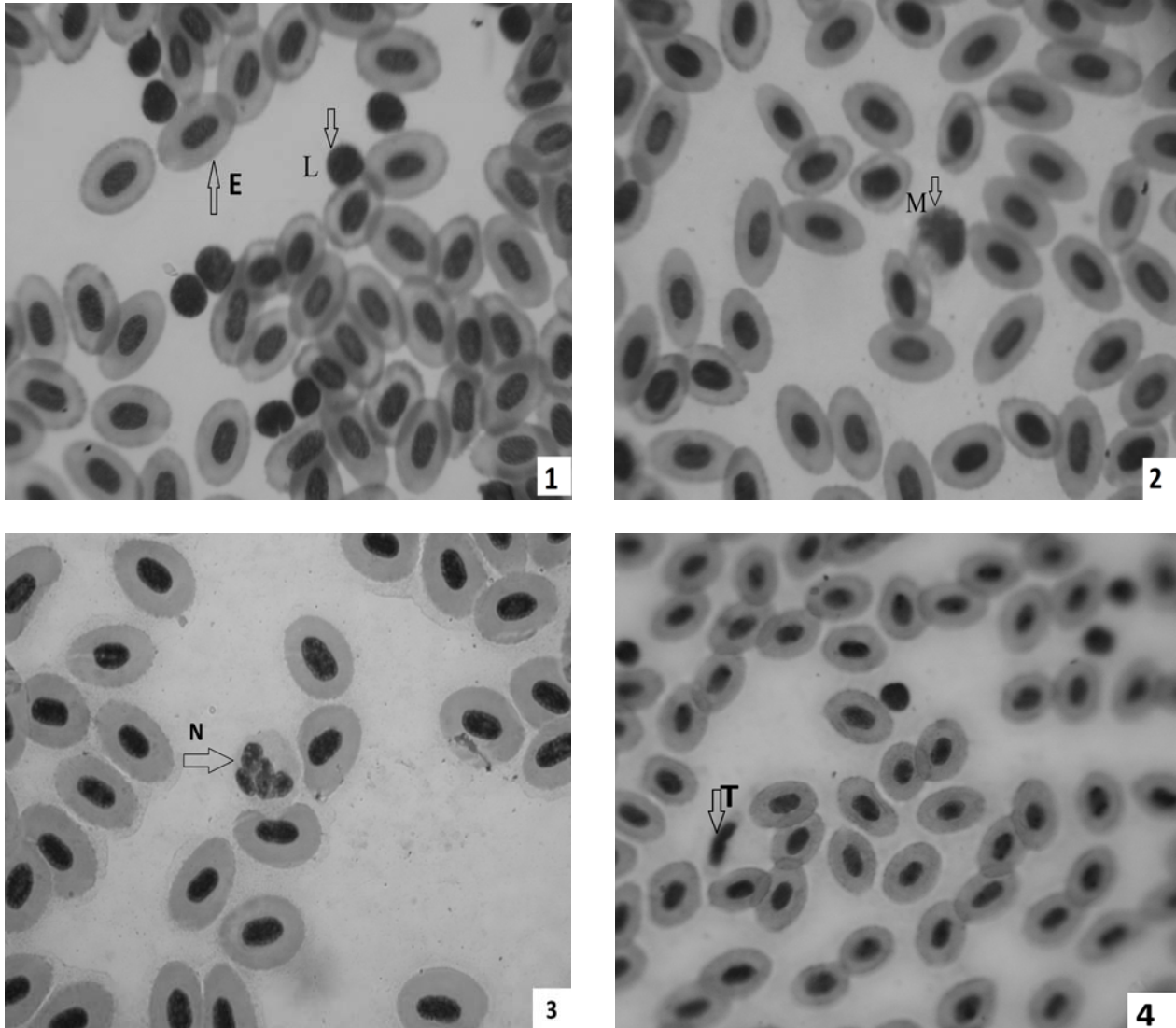
rainbow trout physiological status both qualitatively, in terms of hematological indicators (number of erythrocytes - RBC, hematocrit, hemoglobin, MCV, MCH, MCHC), as well as quantitatively, through technological indicators [5].

The hematological modifications of our studied fish were analyzed in corroboration with the technological factors (stocking density level, water quality) which can influence the metabolic processes. At the end of the experiment, the stocking density reached 6.73 kg/m<sup>3</sup> in DS<sub>1</sub>, 15.35 kg/m<sup>3</sup> in DS<sub>2</sub>, 20.46kg/m<sup>3</sup> in DS<sub>3</sub> and 26.42 kg/m<sup>3</sup> in DS<sub>4</sub>.

For this experiment, the water quality parameters were maintained between optimal limits for *O. mykiss* species; the equipment for water quality conditioning was succesful in terms of treating and reusing the technological water, taking into consideration the fact that water daily losses did not exceed 10% out of the total volume of the system [6]. Previous studies on fish hematology revealed that blood parameters levels could be affected by water temperature and oxygen concentration variations [7]. In the present research, temperature was kept at a constant value during the whole experiment, the mean value being 16.10 $\pm$ 0.07°C and also, dissolved oxygen (DO) mean value was 6.20 $\pm$ 0.58 mg/l<sup>-1</sup>. In order to obtain a detailed image on the changes produced

in leukocyte system, the relative number (percentage) of white cells (leukograma) was correlated with the absolute number of white blood cells (cells/mm<sup>3</sup> blood). Microscopic

examination of blood smears colored with MGG, did not show morphologic changes among leukocytes (Figure 1-4).



**Figure 1-4.** Morphology of circulating blood cell of the rainbow trout, 10 oc x 100 ob, MGG staining: Er-erythrocytes, L-lymphocytes, M- monocyte, N-neutrophils, T-trombocytes

In the present study, the rainbow trout leukograma reflects the predominance of lymphocytes reported to total number of leukocytes, followed by neutrophils and monocytes. No eosinophils or basophils were found in fish from this study. The mean percentage values of the number of different

types of leukocytes (Figure 5) shows differences between experimental variants. Thus it can be observed a slight decrease in the number of lymphocytes, from 97.75±0.35(%) in DS<sub>1</sub> to 96.63±1.25(%) in DS<sub>4</sub>, with increasing the stocking density.

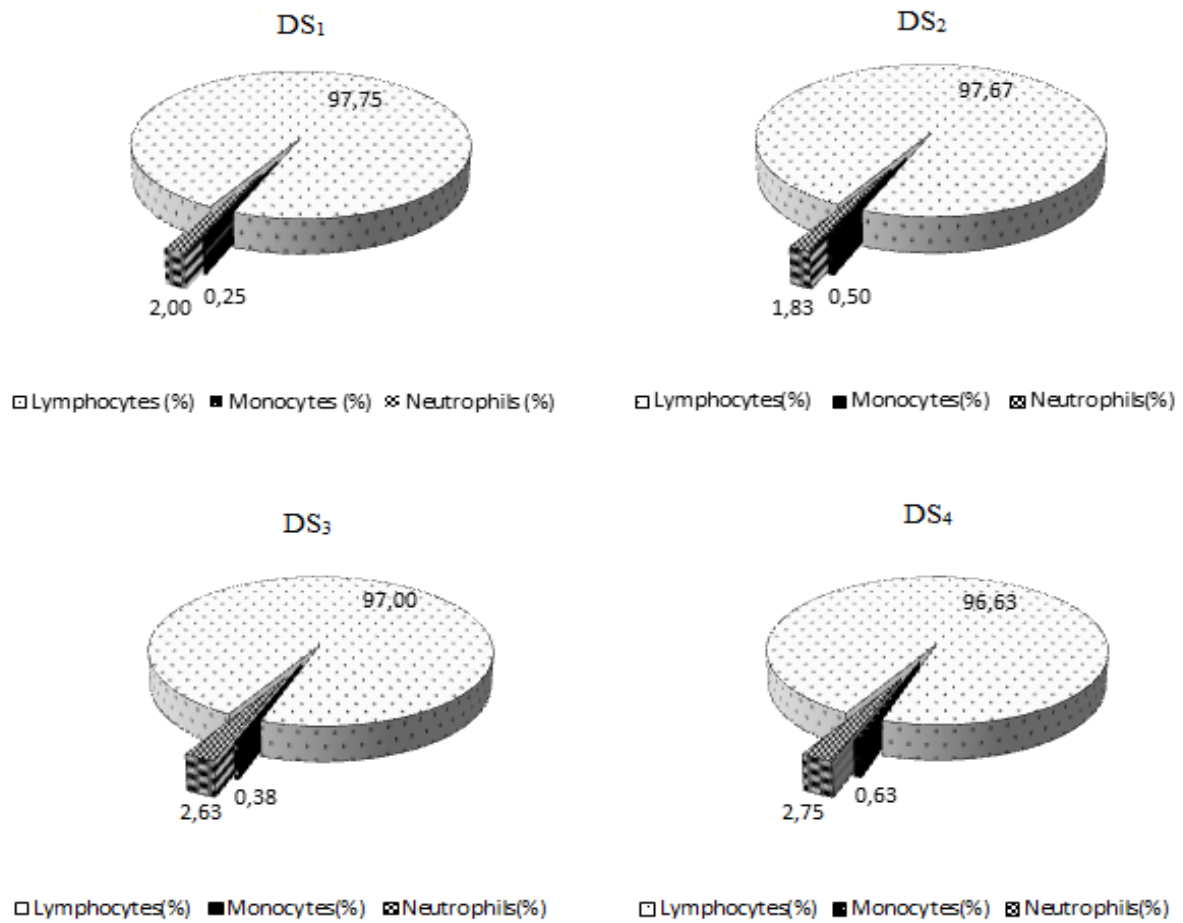


Figure 5. The leukograma of rainbow trout held under different stocking densities

Concerning the relative number of lymphocytes and absolute number of cells reported to the total number of leukocytes per blood volume (mm<sup>3</sup>), it can be observed that they are dominant comparing

to other types of leukocytes. Changes in absolute number of lymphocytes, monocytes and neutrophils are presented in Table 2 (mean±SD).

Table 2. Changes in absolute values of the leukocyte series during experimental period

Cell type		DS <sub>1</sub> (26)	DS <sub>2</sub> (52)	DS <sub>3</sub> (76)	DS <sub>4</sub> (103)
Leukocytes (x10 <sup>3</sup> cel./mm <sup>3</sup> )	Avg±SD	69.79±10.22	69.62±17.46	54.56±17.12	46.34±9.83
Lymphocytes (x10 <sup>3</sup> cel./mm <sup>3</sup> )	Avg±SD	68.24±10.25	68.24±17.21	53.06±17.29	44.80±9.64
Monocytes (x10 <sup>3</sup> cel./mm <sup>3</sup> )	Avg±SD	0.16±0.22	0.09±0.16	0.18±0.24	0.24±0.30
Neutrophils (x10 <sup>3</sup> cel./mm <sup>3</sup> )	Avg±SD	1.4±0.21	1.28±0.59	1.32±0.16	1.30±0.58
Trombocytes (x10 <sup>3</sup> cel./mm <sup>3</sup> )	Avg±SD	2.50±3.54	7.93±2.22	9.38±1.65	8.09±2.27

By analyzing the variation of absolute number of leukocytes (x10<sup>3</sup>cel./mm<sup>3</sup>), reported to DS<sub>1</sub>, we

observe a slight decrease, with 50.61% in DS<sub>4</sub> experimental variant, 27.91% in DS<sub>3</sub> and

approximately 0.25% in DS<sub>2</sub> case. The reduce of leukocytes number (leukopenia) is a hematological parameter known as a stress indicator [8]. Leucocytes total number seems to have a wide range of variation, from 2000 to 63000/mm<sup>3</sup>, for brown trout *S. Trutta*.

Regarding the absolute number of lymphocytes, similar values were recorded in DS<sub>1</sub> and DS<sub>2</sub> experimental variants (68.24x10<sup>3</sup>cel./mm<sup>3</sup>), at DS<sub>3</sub> it decreased to 28.60% and respectively to 52.32%, in DS<sub>4</sub> case. This finding agrees with Wendelaar [9], who reported that stress cause a reduction of lymphocytes in peripheral blood. Pickering and Pottinger [10] observed a reduction of lymphocytes at age-I brown trout *Salmo trutta* and at rainbow trout, as the density increased from 18 to 123 kg/m<sup>3</sup>, without affecting neutrophils number. Lymphocytes are key white blood cells, involved with the coordination of specific and nonspecific immune components and their abundance in the blood is usually a long-term (chronic) response. At salmonids, lymphocytes densities range between 1.6 - 4.57 cells per 100 erythrocytes [11]. Regarding the neutrophils number, in our experiment we registred a slight decrease with the increseang of stocking density (from 1.4±0.21 x10<sup>3</sup>cel./mm<sup>3</sup> to 1.28±0.59x10<sup>3</sup>cel./mm<sup>3</sup> in DS<sub>2</sub>).

The monocyte reaction showed differences between stocking densities, so the absolute number of monocytes at DS<sub>1</sub> variant was 0.16±0.22x10<sup>3</sup>cel./mm<sup>3</sup>, increasing with 0.39% in DS<sub>4</sub> case. As it can be observed from Table 2, by studying the average of absolute values of trombocytes differences between experimental variants an be seen. Fish trombocytes are cells of the immune system, produced by spleen [12], involved in nonspecific defense mechanisms, playing a real protective barriers role. Thus, the significant increasing of trombocytes absolute number, from 2.50±3.54 cel/mm<sup>3</sup> at DS<sub>1</sub> to a value of 9.38±1.65 at / mm<sup>3</sup> in case of DS<sub>3</sub>, indicate the stressful effect of stocking density.

During stress periods, due to increased corticosteroid hormones (cortisol, catecholamines), the number of trombocytes in blood increase and clotting time decreased [13].

#### 4. Conclusions

The hematological assessment of intensively farmed fishes is an integral part of their health status evaluation. In conclusions, the results of the present study shows that rainbow trout held at high stocking densities appear to be experiencing chronic stress, as indicated by the major changes in the leukocyte system. A slight trend of reducing the lymphocytes with increasing stocking density can be observed. This demonstrates that stress inducing by high stocking densities does change the proportion and number of fish blood leukocytes. The primary consequence of a low number of lymphocyte is immunosuppression, resulting an increased susceptibility to desease. Further experiments are required for studying whether high stocking densities affects the ability of fish to resist to additional stressors.

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