

Research article

## EFFECTS OF TRANSPORT AND HOLDING STRESS ON PRUSSIAN CARP (*Carassius gibelio*, Bloch, 1782.) LEUKOGRAM PATTERN

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Stress inevitably occurs during any fish handling and manipulation in culturing, research, or clinical examination situations that require capture and removal of fish from water. Different stress factors can affect the changes in the relative numbers and function of cells of the fish immune system. Catching, transportation and over-crowding caused stress-induced changes in the total number of leukocytes and thrombocytes, as well as changes in the leukocyte formula in Prussian carp (*Carassius gibelio*, Bloch, 1782) presented here. Cytochemical characterization of leukocyte cell types was performed by applying Myeloperoxidase (MPO), Periodic Acid Schiff (PAS) and Sudan Black B (SBB) staining of Prussian carp blood smears. Cytochemical characterization is a rapid and efficient method for white leukocyte differentiation and insight in their functional status. Comparison and analysis in Prussian carp hematological parameters from fish with and without exposure to stressful conditions such as capture, manipulation, transport and holding, revealed significant differences between stressed and non-stressed fish. Significant reduction in the total number of thrombocytes and lymphocytes and the increase in total neutrophil count were observed in stressed animals. However, differences in total leukocyte number and the number of monocytes were not observed. Deviations from the estimated reference intervals for Prussian carp hematological parameters clearly indicated the presence/absence of a stress reaction and to some extent its intensity. Estimated reference intervals and characterization of morphological and cytochemical appearance of blood cells form a solid basis for further research of the cellular immune function and hematology changes in Prussian carp.

**Keywords:** cytochemical characterization, leukocytes, Prussian carp, stress

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## INTRODUCTION

Intensive production technology is the dominant approach in modern aquaculture. However, together with industrial and communal wastewater, and runoff from agricultural areas, effluent from intensive aquaculture can contribute to increases of pollution in open waters. Therefore, there is a need to consider the influence of the polluted environment as a stress factor on fish health. Conditions of acute or chronic stress can cause severe consequences, including decrease in production parameters and deteriorated health with increased mortalities. Fishes sensitivity and susceptibility to environmental stress is high compared to terrestrial animals due to the very close relationship with the water in which they live. It is known that in well-managed ponds mortalities due to acute stress are very rare. It is also known that chronic stress can be responsible for the increased susceptibility to diseases, metabolic changes and changes in energy utilization leading to reduced growth, suppression of the immune response, and inhibition of gonadal maturation and ovulation [1].

In European warm water pond aquaculture, the Prussian carp (*Carassius gibelio*, Bloch, 1782) is often present as an accompanying species. Their natural habitats are still waters and lowland rivers, and ecologically they are tolerant to pollution and low oxygen concentrations. Presently, it is the most dominant fish species in lentic and slow running aquatic habitats [2]. Prussian carp has been shown to both have resilience and be responsive to different external factors that can lead to increased stress. Such a combination of eco-physiological characteristics indicates the potential for this species to be used as a model for environmental contaminant effect on fish populations [3]. However, the availability of literature data on the values of hematological parameters, mechanisms of stress responses to various factors and their connections with immune responses of Prussian carp are limited [3].

Hematological methods are relatively simple and effective tools used in fish health assessment. Processing and analysis of blood samples can be routinely performed in veterinary and research laboratories. Analytical approaches in routine clinical evaluation of blood smears (cell morphology and count) do not require specialized equipment other than a staining station and a microscope. However, expert knowledge of the hematology of the given species, as well as at least some information on the base reference values of the observed parameters is mandatory. While leukograms can be used to indicate the presence of a disorder, in this case stress, there are definite limitations of the technique in the determination of the underlying cause of the observed abnormal findings. The presence of acute, or sometimes chronic, stress can be detected in fish by monitoring changes in the values of hematological parameters. Acute stress in fish and other animals is known to cause neutrophilia and lymphopenia. Functional consequences of stress on leukocytes include inhibition of phagocytosis, reduction in the ability to synthesize antibodies and inhibition of immunological memory formation. The consequences of stress can be observed for a few days after the removal of stressors [4].

Differences in total white blood cell counts can be attributed to many factors, both biotic (such as age, gender, sexual maturity, pathogens) and abiotic (water temperature, pH, dissolved oxygen content, season). Technical issues with the slide preparation, staining methodology, and experience in counting can also interfere with the standardized evaluation of fish blood smears. Characterization of fish leukocytes, especially granulocytes, has not yet been systematized, and with interchangeable use of morphological versus functional characteristics to define different cell types, it may lead to confusion. Furthermore, with significant biological end evolutionary diversity of over 34,000 fish species [5], it is difficult to develop a classification system to accommodate the variability in the shape of the leukocyte, forms and function. Some species have only neutrophilic granulocytes and others only heterophils, while in a few species both cell types can be present [6], reinforcing the need for experienced evaluator and use of cytochemical stains to further delineate clinically relevant morphological and functional characteristics of white blood cells.

Classification of fish leukocytes was frequently performed using criteria related to mammalian leukocytes [7] and several studies indicated that many fish leukocytes have morphological and functional similarities to their mammalian counterparts [8-10]. Within the above-mentioned limitations, leukograms are used in the clinical assessment of health status and stress in fish [11]. As fish leukocytes show variable morphology [12], additional characterization is often required to support accurate classification of blood cell types prior to clinical evaluation and determination of potential for function testing. In such cases, rapid cytochemical staining kits enable the precise identification and differentiation of fish leukocytes based on the presence of certain enzymes in the granules and structural components of the organelle membranes [13]. The purpose of the present study was to characterize the changes in leukogram of Prussian carp exposed to acute stress, using cytochemical stains to improve the accuracy of blood smear counts of selected cell types (granulocytes, lymphocytes and thrombocytes).

## MATERIAL AND METHODS

### Ethical approval

The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. The experiment was approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade and the Ministry of Agriculture, Forestry and Water Management - Veterinary Administration of the Republic of Serbia, decision No. 323-07-01116 / 2023-05 / 1.

### Fish and blood sampling

Prussian carps (*Carassius gibelio*, 20 individual fish) were caught by netting and angling for health examination by licensed veterinarians. Veterinary health inspections of the

ponds and the behavior of the fish were carried out during sample collection. The fish were anesthetized using buffered solution of tricaine-methane-sulfonate (pH 7.4; MS-222, Sigma, St. Louis, Missouri, USA) at a concentration of 75 mg/L for 2 minutes. Blood samples were collected from all sampled and anesthetized fish by caudal vein puncture immediately after capture. Pasteur pipettes ( $\varnothing$  6x150 mm, Soda-lime-glass) with anticoagulant lithium heparin were used for blood collection from the caudal artery and vein. Blood smears were immediately prepared in triplicate for each type of staining, air-dried, stored in a box to prevent exposure to light and kept at 4 °C until staining (<72h). The procedure of blood sampling and making blood smears lasted <2 minutes per fish. Fish were transported live to the Laboratory for fish diseases, Faculty of Veterinary Medicine, University of Belgrade, basic morphological parameters of the examined specimens were measured (total length, weight) and fish placed in aquaria with strong aeration, simulating prolonged transport conditions. After 24 hours (next day), blood was again collected from the same individuals for the preparation of new blood smears as described above. Following anesthesia for blood collection, fish were killed by an overdose of anesthetic (1 g L<sup>-1</sup> of buffered tricaine-methyl-sulfonate solution, MS222, Sigma, Germany) followed by a sharp blow on the head [14].

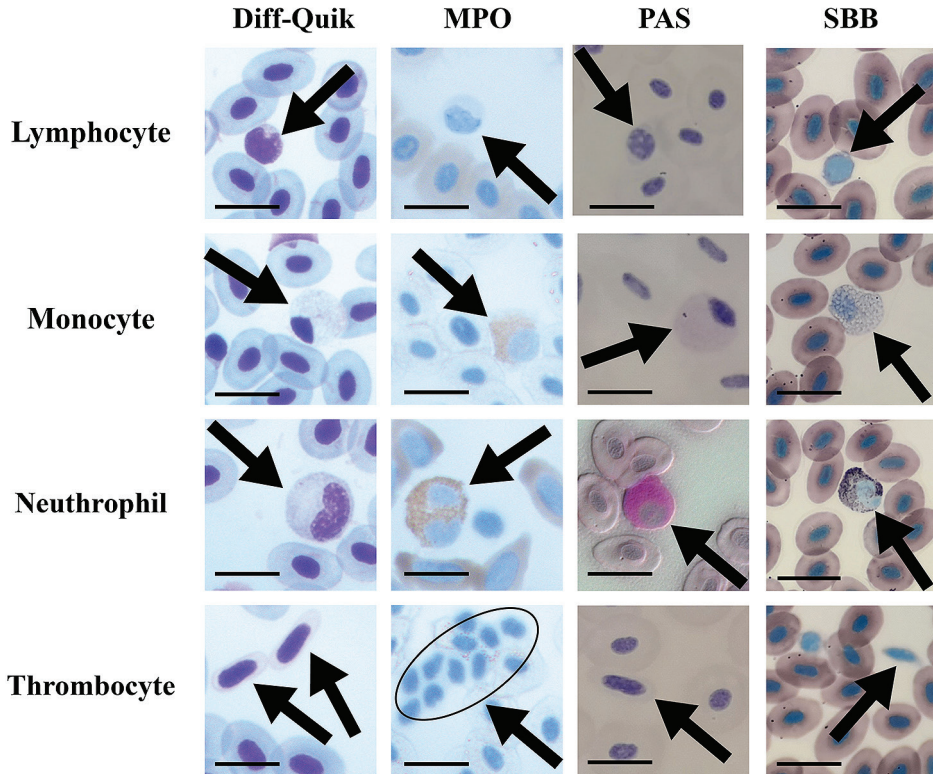
Diff Quick<sup>®</sup> staining was used to determine morphological characteristic of the leukocytes on the blood smears. The following cytochemical kits were used to characterize and differentiate the granulocytes of Prussian carp: myeloperoxidase (Sigma, kit N<sup>o</sup> 391A), Sudan Black B (Sigma, N<sup>o</sup> 380-B) and Periodic acid-Schiff (Sigma 395 B). Staining was performed as per manufacturer's instructions with modifications as per Palic et al. [13]. Stained blood smears were examined using bright field and differential interference contrast microscopy (400-1000 magnification). Granulocytes on blood smears were evaluated based on the cell morphology, and presence or absence of specific chromogens. Presence of lymphocytes, monocytes, thrombocytes and granulocytes was determined.

### **Absolute leucocyte number and leucocyte formula**

The total number of white blood cells, the total number of thrombocytes and the leukocyte formula were determined on blood smears stained with the Diff-Quick<sup>®</sup> method, in the areas of the blood smear where cells only touch each other, without overlap and blank spaces. The total number of leukocytes and thrombocytes was determined by bright field microscopic examination of the blood smear under 400 X magnification. Leukocytes and thrombocytes were counted in 10 fields of view. The total count was divided by total number of fields of view used and multiplied by 2000 [15]. Determination of the leukocyte formula was performed on blood smears, observed under a microscope with a magnification of 1000 X. Minimum of 200 leukocytes were counted on the smear, recording each type of leukocyte separately, with special attention to differentiate thrombocytes from small lymphocytes (Figure 1). Calculation of the absolute number of individual types of leukocytes was determined

from the total number of leukocytes and their differential ratio in percentages using the following formula:

$$\frac{\text{total number of leukocytes /mL blood} \times \% \text{ one type of leukocyte}}{100}$$



**Figure 1.** Cytochemical staining of peripheral blood cells in the Prussian carp. Lymphocytes were negative in all cytochemical staining applied (MPO, PAS, SBB). Monocytes were barely visibly positive for MPO, weakly positive for PAS, while negative for SBB. Neutrophils were positive for all cytochemical stains applied (MPO, PAS, SBB). Platelets were negative for MPO and SBB, while weakly positive for PAS. Scale bar = 10  $\mu$ m. Magnification is 1,000  $\times$ .

### Statistical analysis

Statistical analysis of the obtained data before and after stress was performed using paired t-test in GraphPad Prism version 8.00 (GraphPad Software, USA) and in MS Excel.

## RESULTS

### **Clinical and pathomorphological examination of fish**

During the clinical examination carried out during fish collection non-specific symptoms of disease such as gathering of fish at the water inlet/outlet, lethargy, swimming in unnatural positions, resting at the pond bottom or rapid fatigue, were not observed. According to the anamnestic data collected, the fish had a good appetite and were within expected production parameters. Mean total length was  $172 \pm 33$  mm and mean weight  $118 \pm 71$  g. All captured fish survived the transport to the laboratory. External examination of fish arriving at the laboratory revealed no obvious signs of illness (wounds, visible secondary bacterial and fungal infections, and cachexia). Necropsy of the specimens after second blood drawing did not reveal any gross signs of disease. Based on the collected information fish were healthy and in good condition.

### **Cytology of blood leukocytes observed with Diff-Quick staining**

On smears stained with the Diff-Quick staining method, it was possible to distinguish white blood cells based on the shape and size of the cells, their color, relative size ratio of the cytoplasm and nucleus, and the presence/absence of cytoplasmic granules.

Prussian carp neutrophils describe as round and relatively large ( $9-12 \mu\text{M}$ ) with moderate nuclear to cytoplasm ratio and cytoplasm containing fine neutrophilic granules. The nucleus is kidney-shaped, sometimes segmented with a moderately coarse nuclear chromatin. Monocytes are round in shape, with basophilic cytoplasm that sometimes contains vacuoles. The nucleus is large, euchromatic in appearance, usually round and placed eccentrically. Lymphocytes in Prussian carp are round and relatively small ( $7-9 \mu\text{M}$ ) cells with basophilic cytoplasm. The nucleus is round and occupies almost the entire cytoplasm. Thrombocytes are predominantly spindle-shaped, but it was also possible to observe some round-shaped cells with a nucleus that follows the shape of the cell (Figure 1).

### **Cytological characteristics of leukocytes subjected to specific staining**

Examination of blood smears stained with MPO and PAS method and Sudan Black B dye under 1000 X magnification with the use of immersion oil revealed the following cytochemical characteristics of Prussian carp white blood cells:

#### **Myeloperoxidase method**

Neutrophilic leukocytes stained with the MPO method show brown-black granulation because they have the enzyme myeloperoxidase in their cytoplasmic granules. Monocytes stained by this procedure stain weakly positive. Lymphocytes and thrombocytes did not show myeloperoxidase activity as evidenced by the negative MPO staining (Figure 1).

### **Periodic acid Schiff method**

PAS staining indicated the presence of glycogen in neutrophil leukocytes. The color of their cytoplasm ranged from purple to red, while the nucleus is colored blue. Prussian carp monocytes contain a significantly smaller number of glycogen granules in their cytoplasm as suggested by the weakly positive reaction during PAS staining. Prussian carp lymphocytes do not contain glycogen granules, supported by negative reaction during PAS staining. Prussian carp thrombocytes contain a significantly smaller number of glycogen granules in their cytoplasm, giving weakly positive reaction during PAS staining (Figure 1).

### **Sudan Black B method**

Sudan Black B staining reveals that membrane of neutrophil granules, and neutrophil leukocytes show blue-black intracellular granulation. Monocytes, lymphocytes and thrombocytes of Prussian carp do not stain with Sudan Black B dye (Figure 1).

### **Stress leukogram in Prussian carp**

Total differential leukocyte counts were analyzed with paired t-test comparing leukocyte counts before and after stress. Total number of leukocytes between Prussian carp that were not exposed to stress factors compared to those exposed to stress was not significantly different ( $p > 0.05$ ), which indicated that the leukopenia (reduction of the total number of leukocytes, not including thrombocytes) in Prussian carp that were exposed to stress factors is not readily observable. However, contrary to this finding, the same test proved a statistically significant decrease in the total number of thrombocytes (significant thrombocytopenia) in Prussian carp who were exposed to stress, compared to those who were not exposed to stress factors ( $p < 0.01$ ) (Figure 2).

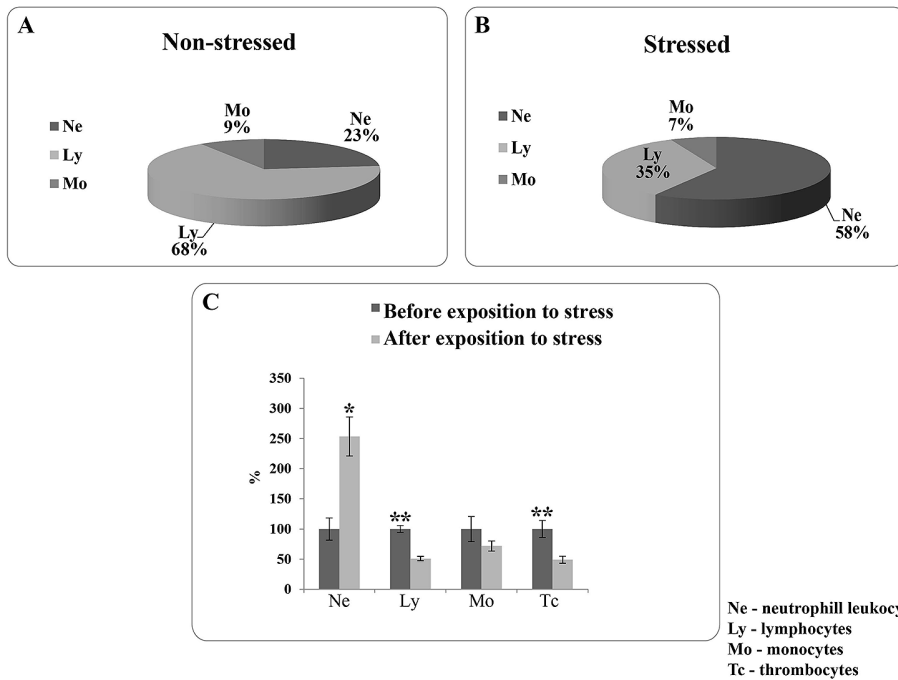
The absolute number of neutrophil leukocytes in Prussian carps that were not exposed to stress was significantly lower compared to stressed fish ( $p < 0.01$ ), indicating acute neutrophilia in Prussian carps exposed to stress factors. Concurrently, statistically significant lymphopenia in Prussian carp was observed in stressed fish ( $p < 0.01$ ). Monocytes showed no statistically significant change between Prussian carp that were not exposed to stress factors compared to those exposed to stress factors ( $p > 0.05$ ) (Table 1).

**Table 1.** Comparative analysis of leukograms in non-stressed and stressed Prussian carp with estimations of respective reference values

	Non-stressed Prussian carp	Stressed Prussian carp
Total leukocyte number/mm <sup>3</sup>	Mean and standard error	13,080.00±818.78
	Minimum count	7,200
	Maximum count	19,000
	95% confidence interval (97.5th and 2.5th percentile)	14,794.00 - 11,366.00
Total thrombocyte count/mm <sup>3</sup>	Mean and standard error	43,040.00±6,061.97
	Minimum count	8,400
	Maximum count	11,600
	95% confidence interval (97.5th and 2.5th percentile)	55,728.00 - 30,352.00
Absolute number of neutrophil leukocytes /mm <sup>3</sup>	Mean and standard error	3,040.00±557.63
	Minimum count	1,028
	Maximum count	11,122
	95% confidence interval (97.5th and 2.5th percentile)	4,207.00 - 1,873.00
Absolute number of lymphocytes /mm <sup>3</sup>	Mean and standard error	8,846.00±499.72
	Minimum count	5,476
	Maximum count	12,350
	95% confidence interval (97.5th and 2.5th percentile)	9,892.00 - 7,800.00
Differential leukocyte count (without thrombocytes)	Mean and standard error	1,194.00±247.38
	Minimum count	288
	Maximum count	4,271
	95% confidence interval (97.5th and 2.5th percentile)	1,711.00 - 675.80
Absolute number of monocytes /mm <sup>3</sup>	Mean and standard error	857.00±97.82
	Minimum count	254
	Maximum count	1,921
	95% confidence interval (97.5th and 2.5th percentile)	1,061.00 - 651.90

Non-stressed control is set as 100% (n=10). Error bars represent standard error and asterisks represent significant difference (\*p<0.01; \*\*p <0.05)





**Figure 2.** Relative abundance of leukocytes in (A) non-stressed and (B) stressed fish; (C) Changes in relative abundance of leukocytes in stressed fish compared to non-stressed control. Lymphocytes are the dominant cell group, followed by neutrophils and leukocytes. The least represented cells are monocytes (A); Neutrophil leukocytes dominate, followed by lymphocytes. The percentage of monocytes in Prussian carp exposed to stress is also significantly lower than the percentage of neutrophil leukocytes and lymphocytes (B); A statistically significant increase in the percentage of neutrophil leukocytes ( $p < 0.01$ ) can be observed (\*), as well as a statistically significant ( $p < 0.05$ ) decrease in the percentage of lymphocytes and thrombocytes in Prussian carp exposed to stress compared to Prussian carp that were not exposed to stress (\*\*). No statistically significant difference was found in the percentage of monocytes ( $p > 0.05$ ) (C).

## DISCUSSION

Cytochemical staining kits often target the activity of intracellular/intragranular enzymes or membrane building components such as glycolipids or lipids, therefore staining results can provide an insight into the components of blood cells and give us evidence of their function status and differentiation stages, which is especially vital in fish immunology research [16].

Myeloperoxidase is a crucial component in the catalytic reaction involved in the production of ROI (reactive oxygen intermediates), specifically hydrogen peroxide from superoxide, which plays an important role in early innate clearance of bacterial pathogens [17]. The examination of peroxidase reactivity of leukocytes was carried out in different fish species [4,18,19]. Among myeloperoxidase (MPO) detection in blood

cells of these fish species, most neutrophils primary granules were MPO positive, supporting their primary phagocytic and killing role in innate immune responses. The cytochemical findings in the study of phagocytes implied that upon phagocytosis, the peroxidase in the phagosomes was part of a microbicidal system operating as a host defense mechanism against bacterial infection. Researchers believe that the presence of peroxidase indicates the potential for measuring oxidative bursts and degranulation in neutrophil primary granules, such as those in Arctic grayling, cutthroat trout and June sucker [4].

The positive finding of myeloperoxidase staining of neutrophil leukocytes in our research confirms findings in other fish species [9,12,13,20,21] and demonstrates very similar cytochemical characteristics to mammalian neutrophils.

A positive finding of myeloperoxidase staining is not exclusively related to neutrophil leukocytes, but can also be seen in monocytes, although it is significantly less intense. Monocytes can have a higher level of peroxidase activity [22], when the number of neutrophils is also increased [23]. In our research, Prussian carp monocytes were not or barely visible positive on MPO staining.

Periodic acid–Schiff (PAS) and Sudan Black B (SBB) staining are an indication of glycogen in the cellular organelle membranes (e.g. in granules). In fact, except glycogen, glycoproteins and polysaccharides are also observed by PAS reaction. Glycogen in cells is believed to be an energy source for both aggregatory and phagocytic abilities [24,25].

Neutrophils in human and mammals are mainly involved in the phagocytosis and degradation of invading microorganisms [16,26,39]. Strongly positive reactions for SBB and PAS indicated that the neutrophils of Prussian carp are similar to those of mammals, which have strong phagocytic and bactericidal abilities.

Monocytes of tilapia [22], Murray cod [25], *S. prenanti* [28], and piebald naked carp [29], were positive for PAS but negative for SBB staining, indicating that fish monocytes have phagocytic and antigen-presenting functions. In our study monocytes were only weakly positive and also SBB negative (Figure 1).

Lymphocytes in Prussian carp were negative for PAS (Figure 1), as reported for multiple species including channel catfish [24], four freshwater teleosts [30], the South American catfish [31], and fat snook [32].

Thrombocytes are described as the most abundant blood cells after erythrocytes, and the thrombocyte count is frequently used as an indicator of health status because they are mainly involved in forming defensive barriers [33]. Fish thrombocytes participate in hemostatic processes and in the immune response [34,35]. There are no clear tendencies of changes in the total number of thrombocytes in fish exposed to different stress factors. Under the influence of various stress factors, blood coagulation is often accelerated, but this is not always accompanied by an increase in the total number of thrombocytes. The decrease in the total number of thrombocytes in Prussian carp

exposed to stress is observed [23]. Contrary to this finding, different stress factors significantly affect the increase in the number of thrombocytes in the peripheral blood of channel catfish [24].

Studies have investigated whether thrombocytes in some fish have phagocytic abilities [24,25,29,32,36]. However, the mechanism for killing pathogens after phagocytosis remains unknown [37]. Some researchers believe that fish thrombocytes have a nonenzymatic mechanical phagocytic ability because no enzyme activity was detected in thrombocytes in Murray cod [25]. The presence of MPO was closely related to the ability of cells to produce respiratory bursts.

Glycogen in thrombocytes represents a source of energy for them and those thrombocytes play a role in phagocytosis of foreign substances. This process requires the consumption of energy, both from endogenous and exogenous sources [38].

In thrombocytes of channel catfish and goldfish, the reaction of blood cells in PAS staining was weakly positive [24], while in rainbow trout and Pacific salmon it was negative [10]. We have demonstrated a weakly positive reaction of thrombocytes in PAS staining in the Prussian carp, which is in accordance with the findings in the goldfish.

There are differences in the findings of PAS and MPO staining even in fish species belonging to the same family, thrombocytes in grass carp were positive in both the PAS reaction and MPO, and the crucian carp's thrombocytes were positive for the PAS reaction but were negative for MPO activity [39]. The finding in Prussian carp is the same as in crucian carp.

Positive Sudan Black B staining was observed in thrombocytes while in our study thrombocytes were SBB negative [40].

The finding of basophilic and eosinophilic leukocytes in the blood of fish is quite rare [12,41] and our research confirms this claim.

Stress-induced hematological changes depend on type of stressor, its magnitude and time of action [42]. Leukocyte count (WBC) is an important parameter in the assessment of the immune status in vertebrates [43]. Stress may affect white blood parameters. Short-term stress sometimes results in an increase in WBC but chronic and/or strong stress usually causes leukopenia [44]. In the course of our research, we found out that the total number of leukocytes in the peripheral blood of Prussian carp exposed to stress factors was only slightly lower compared to the values of the group of fish that were not exposed to stress. A tendency to increase the total number of leukocytes may indicate an increased protective function of blood in the body of the Prussian carp under the stress as action of chemical toxicants [45].

Differential leukocyte counts belong to important characteristics of the health state of fish and in many cases they are also helpful in evaluating the immune system and variations in the proportion of these cells are considered normal [9]. The neutrophils and lymphocytes numbers are affected by stress in opposite directions [46]. Generally,

a fish stress leukogram reflects leukopenia with lymphopenia and granulocytosis. Such changes may persist for several days after the stressor is removed [11].

Teleost neutrophils are phagocytes and typically the first leukocytes recruited to an inflammatory site by chemokines [47] and are capable of eliminating pathogens through multiple complementary mechanisms. Activated neutrophils become powerful killers, utilizing toxic intracellular granules, the production of reactive oxygen species (ROS) [48], and secrete neutrophil extracellular traps (NETs) which consist of antimicrobial granular proteins that prevent the dissemination of invading pathogens [49,50].

The characteristic mark of the stress-leukogram in fish is lymphopenia and neutrophilia [51,52]. The results obtained in our research are in accordance with the results of these authors, and are in the contrary to studies claiming that the numbers of circulating lymphocytes and neutrophils remain unchanged after exposure to stress [30].

Evaluation of the leukocyte formula cannot be equated with measuring the degree of immune response *per se*, but can be determined more directly using other methods [53]. Changes in the total number of circulating leukocytes, their redistribution and quantitative shifts within the leukocyte formula are the most frequently observed effects in fish exposed to stress.

## CONCLUSIONS

Rapid cytochemical characterization of fish blood cell types with human-based staining kits proved to be an effective method for differentiating Prussian carp leukocytes. The presented information about morphological appearance and leukocyte counts provide a basis for further research into the function and changes of blood cells of Prussian carp exposed to variable environmental stress factors.

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### Authors' contributions

AK, RA, MM, LM and PD participated in the design of the study. AK collected samples for analysis. AK, LM and PD carried out the laboratory analysis, and RA performed the statistical analysis. AK, PD and RA drafted the manuscript. MM and LM participated in the coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

### Declaration of competing interests

The author(s) declare that they have no competing interests.

## REFERENCES

1. Jeremić S, Jakić-Dimić D, Veljović Lj: *Citrobacter freundii* as a cause of disease in fish. Acta Vet-Beograd 2003 53: 399-410.
2. Lusk S, Lusková V, Hanel L: Alien fish species in the Czech Republic and their impact on the native fish fauna. Folia Zool 2010 59:57-72.
3. Topić Popović N, Strunjak-Perović I, Barišić J, Kepec S, Jadan M, Beer-Ljubić B, Matijatko V, Palić D, Klobučar G, Babić S, Gajdoš Kljusurić J, Čož-Rakovac R: Native Prussian carp (*Carassius gibelio*) health status, biochemical and histological responses to treated wastewaters. Environ Pollut 2016 218:689-701.
4. Palić D, Beck SL, Palić J, Andreasen BC: Use of rapid cytochemical staining to characterize fish blood granulocytes in species of special concern and determine potential for function testing. Fish Shellfish Immunol 2011 30:646-652.
5. Froese, R. and D. Pauly. Editors. 2022. FishBase. World Wide Web electronic publication. www.fishbase.org, version (08/2022).
6. Witeska M, Kondera E, Ługowska K, Bojarski B: Hematological methods in fish – Not only for beginners, Aquaculture 2022 547:737498.
7. Ellis AE: The immunology of teleosts. In: Roberts RJ, editor. Fish pathology. 3rd ed. London/Edinburgh/New York/Philadelphia/St. Louis/Sydney/Toronto: W. B. Saunders; 2001. p. 133e50.
8. Groff JM, Zinkl JG: Hematology and clinical chemistry of cyprinid fish. Common carp and goldfish. Vet Clin North Am Exot Anim Pract 1999 2:741e76.
9. Tavares-Dias M, Moraes FR: Morphological, cytochemical, and ultrastructural study of thrombocytes and leukocytes in neotropical fish, *Brycon orbignyanus Valenciennes*, 1850 (*Characidae*, *Bryconinae*). J Submicrosc Cytol Pathol 2006 38:209e15.
10. Zinkl JG, Cox WT, Kono CS: Morphology and cytochemistry of leucocytes and thrombocytes of six species of fish. Comp Haematol Int 1991 1:187-195.
11. Clauss TM, Dove ADM, Arnold JE: Hematologic disorders of fish. Vet Clin North Am Exot Anim Pract 2008 11:445e62.
12. Ravi V, Venkatesh B: Rapidly evolving fish genomes and teleost diversity. Curr Opin Genet Dev 2008 18:544e50.
13. Palić D, Andreasen CB, Frank DE, Menzel BW, Roth JA: Gradient separation and cytochemical characterisation of neutrophils from kidney of fathead minnow (*Pimephales promelas* Rafinesque, 1820). Fish Shellfish Immunol 2005 18:263-267.
14. AVMA Guidelines for the Euthanasia of Animals: 2020 Edition, <https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf>
15. Fudge AM, (ed): Laboratory Medicine Avian and Exotic Pets. Philadelphia, W.B. Saunders. 2000, pp 9-15.
16. Zheng ZX, Tang Y, Fang J, Peng X, Fan JD, Cui HM, Yang LZ: Ultrastructural and cytochemical properties of peripheral blood cells of piebald naked carp (*Gymnocypris eckloni*). Anat Histol Embryol 2016 46:17-24.
17. Espelid S, Løkken GB, Steiro K, Bøgdal J: Effects of cortisol and stress on the immune system in Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol 1996 6: 95–110.
18. Bianchi MB, Jerônimo GT, Pádua SB, Satake F, Ishikawa MM, Tavares-Dias M, Martins ML: The hematological profile of farmed *Sorubim lima*: reference intervals, cell morphology and cytochemistry. Vet Arh 2014 84:677–690.

19. Da Silva WF, Egami MI, Santos AA, Antoniazzi MM, Silva M, Gutierrez RC, Paiva MJR: Cytochemical, immunocytochemical and ultrastructural observations on leukocytes and thrombocytes of fat snook (*Centropomus parallelus*). *Fish Shellfish Immunol* 2011 31:571–577.
20. Magnadóttir B: Innate immunity of fish (overview). *Fish Shellfish Immunol* 2006 20:137–151.
21. Stosik M, Deptula W, Trávníček M, Baldy-Chudzik K: Phagocytic and bacterial activity of blood thrombocytes in carps (*Cyprinus carpio*). *Vet Med* 2002 47:21–25.
22. Stosik M, Tokarz-Deptula B, Deptula W: Characterisation of Thrombocytes in Osteichthyes. *J Vet Res* 2019 63:123–131.
23. Shigdar S, Cook D, Jones P, Harford A, Ward AC: Blood cells of Murray cod *Maccullochella peelii peelii* (Mitchell). *J Fish Biol* 2007 70:973–980.
24. Langermans JA, Hazenbos WL, van Furth R: Antimicrobial functions of mononuclear phagocytes. *J Immunol Methods* 1994 174:185–194.
25. Imagawa T, Hashimoto Y, Kitagawa H, Kon Y, Kudo N, Sugimura M: Morphology of blood cells in carp (*Cyprinus carpio* L.). *Jpn J Vet Sci* 1989 51:1163-1172.
26. Zhang F, Feng R, Fang W, Shi Y, An L, Yang G: Cytochemical characterization of peripheral blood cell populations of two Cyprinidae, *Carassius auratus* and *Ctenopharyngodon idellus*. *Anat Histol Embryol* 2019 48:22-32.
27. Tripathi NK, Latimer KS, Burnley VV: Hematologic reference intervals for koi (*Cyprinus carpio*), including blood cell morphology, cytochemistry, and ultrastructure. *Vet Clin Pathol* 2004 33:74–83.
28. Tavares-Dias M: A morphological and cytochemical study of erythrocytes, thrombocytes and leukocytes in four freshwater teleosts. *J Fish Biol* 2006 68:1822–1833.
29. Burgos-Aceves MA, Lionetti L, Faggio C: Multidisciplinary haematology as prognostic device in environmental and xenobiotic stress-induced response in fish. *Sci Total Environ* 2019 670:1170-1183.
30. Wang Z, Lin L, Chen W, Zheng X, Zhang Y, Liu Q, Yang D: Neutrophil plays critical role during *Edwardsiella piscicida* immersion infection in zebrafish larvae. *Fish Shellfish Immunol*, 2019 87:565-572.
31. Bojarski B, Kondera E, Ługowska K, Witeska M: Differences in hematological values of common carp between cardiac and venous blood. *Bull Eur Ass Fish Pathol* 2018 38:234–239.
32. Tort L: Stress and immune modulation in fish. *Dev Comp Immunol* 2011 35:1366–1375.
33. Kurchenko OV, Sharamok ST: The Hematological Parameters of the Prussian Carp (*Carassius gibelio*, (Bloch, 1782)) Under the Zaporizhian (Dnipro) Reservoir Conditions. *Turk J Fish & Aquat Sci* 2020 20:807-812
34. Davis AK, Maney DL, Maerz JC: The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct Ecol* 2008 22:760-772.
35. Katzenback BA, Belosevic M: Isolation and functional characterization of neutrophil-like cells from goldfish (*Carassius auratus* L.) kidney. *Dev Comp Immunol* 2009 33: 601-611.
36. Flerova EA, Balabanova LV: Zhurnal evoliutsionnoi biokhimii i fiziologii, 2013, 49:162–171.
37. Palić D, Ostojić J, Andreasen CB, Roth JA: Fish cast NETs: Neutrophil Extracellular Traps are Released from Fish Neutrophils. *Dev Comp Immunol* 2007 31:805-816.

38. Pijanowski L, Golbach L, Kolaczowska E, Scheer M, Verburg-van Kemenade BM, Chadzinska M: Carp neutrophilic granulocytes form extracellular traps via ROS-dependent and independent pathways. *Fish Shellfish Immunol* 2013 34:1244–1252.
39. Pulsford AL, Lemairegony S, Tomlinson M, Collingwood N, Glynn PJ: Effects of acute stress on the immune system of the Dab, *Limanda limanda*. *Comp Biochem Physiol Part - C: Toxicol Pharmacol* 1994 109:129–139.
40. Bodammer JE, Robohm RA: Ultrastructural observations on the phagocytic behavior of winter flounder *Pleuronectes americanus* peritoneal neutrophils and macrophages in vivo. *Dis Aquat Org* 1996 25:197–208.
41. Fang J, Chen K, Cui HM, Peng X, Li T, Zuo ZC: Morphological and cytochemical studies of peripheral blood cells of *Schizothorax prenanti*. *Anat Histol Embryol* 2014 43:386–394.
42. Rieger AM, Barreda DR: Antimicrobial mechanisms of fish leukocytes. *Dev Comp Immunol* 2011 35:1238–1245.
43. Prokopowicz Z, Marcinkiewicz J, Katz DR, Chain BM: Neutrophil myeloperoxidase: soldier and statesman. *Arch Immunol Ther Exp* 2012 60:43–54.
44. Petrie-Hanson L, Ainsworth AJ: Differential cytochemical staining characteristics of channel catfish leukocytes identify cell populations in lymphoid organs. *Vet Immunol Immunopathol* 2000 73:129–144.
45. Rønneseth A, Wergeland HI, Pettersen EF: Neutrophils and B-cells in Atlantic cod (*Gadus morhua* L.). *Fish Shellfish Immunol*, 2007 23:493–503.
46. Maule AG, Schreck CB: Changes in numbers of leukocytes in immune organs of juvenile coho salmon after acute stress or cortisol treatment. *J Aquat Anim Health* 1990 2:298–304.
47. Ueda IK, Egami MI, Sasso WS, Matushima ER: Cytochemical aspects of the peripheral blood cells of *Oreochromis (Tilapia) niloticus*. (Linnaeus, 1758) (*Cichlidae, Teleostei*): Part II. *Braz J Vet Res Anim Sci* 2001 38:273–7.
48. Huijie C, Gailing Y, Jianguo S, Xiaoling L: Hematological analysis of *Ctenopharyngodon idella*, *Megalobrama amblycephala* and *Pelteobagrus fulvidraco*: Morphology, ultrastructure, cytochemistry and quantification of peripheral blood cells. *Fish Shellfish Immunol* 2019 90:376–384.
49. Witeska M: Stress in fish – hematological and immunological effects of heavy metals. *Electron J Ichthyol* 2005 1:35–41.
50. Tavares-Dias M, Moraes FR: Leukocyte and thrombocyte reference values for channel catfish (*Ictalurus punctatus* Raf.), with an assessment of morphological, cytochemical, and ultrastructural features. *Vet Clin Pathol* 2007 36:49–54.
51. Shigdar S, Harford A, Ward AC: Cytochemical characterisation of the leucocytes and thrombocytes from Murray cod (*Maccullochella peelii peelii*, Mitchell). *Fish Shellfish Immunol* 2009 26:731–736.
52. Azevedo A, Lunardi LO: Cytochemical characterization of eosinophilic leukocytes circulating in the blood of the turtle (*Chrysemys dorsignib*). *Acta Histochem* 2003 105:99–105.
53. Wedemeyer G.A., Barton B.A., McLeay D.J., 1990, Stress and acclimation, *Methods for Fish Biology* (eds C.B. Schreck & P.B. Moyle), American Fisheries Society, Bethesda, MD, 451–489.

## **UTICAJ STRESA UZROKOVANOG TRANSPORTOM I MANIPULACIJOM NA LEUKOGRAM BABUŠKE (*Carassius gibelio*, Bloch, 1782.)**

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Stres je nerazdvojni deo akvakulture, istraživanja, kliničkog pregleda, kao i svih ostalih manipulacija ribom, pošto svi postupci zahtevaju vađenje ribe iz vode. Različiti faktori stresa imaju uticaj na zastupljenost i funkcije ćelija imunskog sistema. Našim istraživanjima bile su obuhvaćene promene ukupnog broja leukocita i trombocita, kao i promene u leukocitarnoj formuli kod babuški (*Carassius gibelio*, Bloch, 1782.) koje su izložene uticaju stresa usled izlovljavanja, manipulacije, transporta i prenaseljenosti. Posebna pažnja posvećena je citohemijskoj karakterizaciji ovih tipova ćelija kod babuški primenom mijeloperoksidaza (MPO), *Periodic acid Schiff* (PAS) i *Sudan Black B* (SBB) bojenja. Poređenje i analiza ispitivanih parametara kod riba koje su bile izložene faktorima stresa sa onima koje nisu, ukazuje na postojanje značajnih razlika pojedinih parametara. Posle izlaganja stresu došlo je do značajnog smanjenja ukupnog broja trombocita i limfocita a povećanja kod broja neutrofila. Nisu primećene značajne razlike u ukupnom broju leukocita kao ni u broju monocita. Dobijeni referentni intervali i morfološki izgled obezbeđuju osnovu za dalja istraživanja funkcije i promene krvnih ćelija babuški dok brza citohemijska bojenja predstavljaju efikasne metode za diferencijaciju različitih tipova leukocita kod riba. Odstupanje od utvrđenih vrednosti za pomenute hematološke parametre omogućilo je da se utvrdi prisustvo stresne reakcije i delimično njen intenzitet.