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Original Scientific Article

HEMATOLOGICAL IMPORTANCE OF PSEUDOEOSINOPHILIC GRANULOCYTES IN ACCLIMATION OF COMMON CARP (*Cyprinus carpio* Linnaeus, 1758)

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ABSTRACT

Adaptation mechanisms as response to water content, oxygen level and pollutants are very important and they can be interpreted by hematological analysis. The aim of this study was the analysis of hematological and immune adaptations of common carp (*Cyprinus carpio* Linnaeus, 1758) to thermal stress. All specimens were divided into a control and experimental group. The control group of fish was exposed to a constant water temperature of 10°C. We induced thermal stress in experimental fish by gradually heating water to 28°C, held for 30 minutes and then comparing the obtained results with the control fish. Short-term hyperthermia lead to an increase of the number of leukocytes, especially pseudoeosinophilic granulocytes and monocytes, while the number of neutrophils and lymphocytes was reduced. The analysis of the leukocyte number and differential blood count in the control group showed high individual variation of segmented granulocytes, monocytes and pseudoeosinophilic granulocytes. Statistically significant differences (p=0.00) were found for the white blood cells, nonsegmented neutrophils and pseudoeosinophils between the control and experimental group. The experimental group of males had an increased number of white blood cells, monocytes and pseudoeosinophils, where significant differences were found for nonsegmented and total neutrophils and also for pseudoeosinophils (p=0.00), lymphocytes (p=0.01) and monocytes (p=0.03). Females had an increased total number of white blood cells, lymphocytes, monocytes and pseudoeosinophils, while significant differences (p=0.00) were obtained in the number of white blood cells, nonsegmented and total neutrophils and pseudoeosinophils between the control and experimental group. Adaptation mechanisms in carp after water temperature heating are mostly reflected in the increase of pseudoeosinophils and the decrease of neutrophils.

Key words: *Cyprinus*, pseudoeosinophilic granulocytes, acclimation, stress

INTRODUCTION

Different environmental stress factors affect the fish organism in intensive fish culture. Deleterious stimuli such as temperature changes, variation of pH, decrease in oxygen concentration, increase in ammonia concentration, handling, transport and osmotic changes in water induce stress reaction in fish (1, 2). A stress reaction, which is an integrated response with behavioral, neural, hormonal and physiological elements by the fish organism can cause changes in the fish health status and reduce the resistance to diseases (3, 4). Changes in water quality can lead to significant disturbances in fish, such as various diseases and very significant hematological changes in the blood of fish (5). Cellular stress response is a protective response of individual cells to potentially harmful stimuli from the environment (6). Cellular stress response was first described as a reaction to increased temperature (7). To counter stress, the cells increase the expression of chaperones that help in protein refolding and aggregation alleviating (8). The cell thus provides a short-term protection, leading to a condition called thermotolerance and cells become resistant to a

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variety of negative effects (9, 10). One of the important responses in fish adaptation is the hematological response, especially the number of leukocytes, and it is the first defense line against stress agents (9). The differentiation of the blood cells of fish is much debatable, but there is almost no uniform classification or reference interval, neither for fish families neither for the level of the genus *Cyprinus* (11). Fish blood is very rich in leukocytes, which are divided into granulocytes (neutrophils, eosinophils, basophils) and agranulocytes (lymphocytes and monocytes).

Pseudoeosinophilic granulocytes are known as heterophils (12). They are large round cells, with eccentric round to oval nucleus. Eosinophilic, basophilic and neutrophilic granulation are present in cytoplasm whose number depends on the degree of maturation of the cells. It is important to emphasize the simultaneous presence of granules that have different colors (13, 14). A significant increase of pseudoeosinophils marked as pseudoeosinophilia was recorded during stress (15). Most researches compare the functional significance of pseudoeosinophils with neutrophils despite their different morphological characteristics (16). It has been reported that pseudoeosinophils are functionally heterogeneous cells with combined morphological elements and the roles of other granulocytes. Recent researches show that pseudoeosinophilic granulocytes are present in wild fish, especially in tench (*Tinca tinca* Linnaeus, 1758) and sporadically in European chub (*Squalius cephalus* Linnaeus, 1758) (17). It is interesting that crucian carp (*Carassius carassius* Linnaeus, 1758) does not have pseudoeosinophilic granulocytes in their blood, nor does the rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) in the wild (18). Artificially bred trout does not have the ability for a longer survival rate in warmer water (19).

The aim of this study was the determination of hematological changes in common carp caused by thermal stress and the potential ability of adaptation with emphasis on the number of pseudoeosinophilic granulocytes.

MATERIAL AND METHODS

The study was performed on 49 specimens of common carp (*Cyprinus carpio* Linnaeus, 1758), 22 males and 27 females, sampled in the Jablanica reservoir (Bosnia and Herzegovina) after floods in January, 2015. The reservoir is spread from the Konjic municipality to the municipality of Jablanica (43° 41′ 0″ N, 17° 51′ 0″ E) with an area of 13 km^2 and 270 asl. Fishnets were used for fish sampling. Average length of all fish was 13.61 ± 1.34 cm and average body mass was 25.24 ± 6.96 g. All captured fish survived the transport to the laboratory. Fish were transported to the laboratory using barrels where water was continuously enriched with oxygen. The fish were randomly (with approximately equal gender balance) placed in two aquariums (control and experimental) with a total volume of 100 L, equipped with aerators (CHAMPION CX-0098) and heaters (PEAR AQUATICS Y 978). Adaptation lasted 20 days and during that period temperature was set at 10°C for both groups (Laboratory of Physiology, Faculty of Science, Sarajevo, Bosnia and Herzegovina). All fish were fed with Eco FeedEx C 48/10 (Eco Feed Ltd, Serbia). Water monitoring was done daily and included: changing the filters, analysis of oxygen concentration by Winkler's method (20) and analysis of ammonia concentration by Nessler's method (20). This research included 25 control and 24 experimental specimens.

Control specimens (11 males and 14 females) were kept all the time at 10°C, while the water in the aquarium with experimental specimens (11 males and 13 females) was gradually heated to 28°C and at this temperature fish were kept for 30 minutes (21). After that time, the sampling of blood was performed by cardiac puncture. Before puncture of the heart, the fish (both groups) were anaestethised by isoeugenol in concentration of 40 mg/L (22). Blood sampling was performed by heart puncture with a sharp and sterile needle (1.0 to 1.2 mm) under sterile conditions.

Morphometric measurements

The values of basic morphometric features, total and standard length, as well as the mass were obtained. Technical scales were used for body mass estimation, and the standard and total length were measured by an ichthyometer.

Hematological analysis

The analysis of the hematological parameters was performed with native blood without anticoagulant and included the number of leukocytes (WBC) and the morphological differentiation of leukocytes in peripheral blood: segmented (Seg) and nonsegmented neutrophils (Nonseg), lymphocytes (Ly), monocytes (Mo) and pseudoeosinophils (Pse). The number of leukocytes was determined in a Neubauer chamber (hemocytometer) according to the method of Kekic and Ivanc (23) and morphological differentiation was performed using an Olympus BX41 microscope with Olympus DP12 camera.

Statistical analysis

Data are presented as means ± 1 SD accompanied by minimum, maximum and coefficient of variation (%). The Kolmogorov-Smirnov test was used to estimate the normality of data distribution. Student's *t-test* was use to assess the differences between the groups. Statistical analysis were performed by SPSS (Version 20.0, SPSS, Inc., Chicago, IL, USA).

RESULTS

Mass and morphometric parameters

Descriptive statistics of the morphometric parameters and weight of control and experimental group is presented in Table 1.

The analysis of the leukocyte number and differential blood count in the control fish showed high individual variation of Seg (CV=62.17%), Mon (CV=45.51%) and Pse (CV=61.12%). These variations were characteristic for both males and females. Females had a much higher number of Pse and less Ly compared to males. Number of Seg, Nonseg and Mon was approximately equal in control males and females. Types of leukocytes in common carp are shown in Fig. 1.

Table 1. Standard length, total length and mass of control and experimental common carp

Statistical parameter		Standard length (cm)	Total length (cm)	Mass (g)
Control group (males and females)	Mean	11.53*	13.60**	25.24**
	Standard deviation	1.25	1.33	6.96
	Minimum	9.00	10.60	11.20
	Maximum	13.20	15.60	35.00
	Coefficient of variation - %	10.84	13.60	27.57
Experimental group (males and females)	Mean	12.64*	14.59**	35.25**
	Standard deviation	1.38	1.18	9.61
	Minimum	11.50	12.20	66.00
	Maximum	16.80	17.40	26.10
	Coefficient of variation - %	10.91	8.08	27.26

Note: Asterisk in superscript denotes significantly different mean values between groups (*p≤0.05 and **p≤0.01); p-values obtained by Student's *t-test*

Kolmogorov-Smirnov test showed that all morphometric parameters had normal distribution ($p>0.05$).

Hematological parameters

The analyzed white blood cells parameters of the control group are shown in Table 2.

WBC parameters of the experimental group are shown in Table 3.

The experimental fish that were exposed to increased water temperature showed a wider distribution of the analyzed parameters in relation to the control group. This group was characterised by higher values of the coefficient of variation for

Table 2. Hematological parameters of the control group of common carp

Hematological parameters		WBC x 10^9/l	Seg	Nonseg	Ne	Ly	Mo	Pse
Total	Mean	5.30	3.30	28.64	31.68	52.12	2.41	13.40
	Standard deviation	0.90	2.05	6.66	7.06	7.99	1.10	8.19
	Minimum	4.00	1.00	14.00	14.00	33.00	1.00	3.00
	Maximum	6.90	7.00	40.00	42.00	69.00	4.00	31.00
	Coefficient of variation - %	16.89	62.17	23.24	22.27	15.32	45.51	61.12
Male	Mean	5.24	3.09	30.00	33.09	55.82	2.20	8.91
	Standard deviation	0.96	1.92	7.24	7.22	7.24	1.14	4.09
	Minimum	4.05	1.00	19.00	21.00	47.00	1.00	3.00
	Maximum	6.90	7.00	40.00	42.00	69.00	4.00	16.00
	Coefficient of variation - %	18.35	62.15	24.12	21.81	12.96	51.60	45.85
Female	Mean	5.34	3.50	27.57	30.57	49.21	2.57	16.93
	Standard deviation	0.87	2.24	6.22	6.99	7.54	1.09	8.97
	Minimum	4.00	1.00	14.00	14.00	33.00	1.00	4.00
	Maximum	6.70	7.00	37.00	41.00	61.00	4.00	31.00
	Coefficient of variation - %	16.34	63.89	22.57	22.87	15.31	42.37	53.01

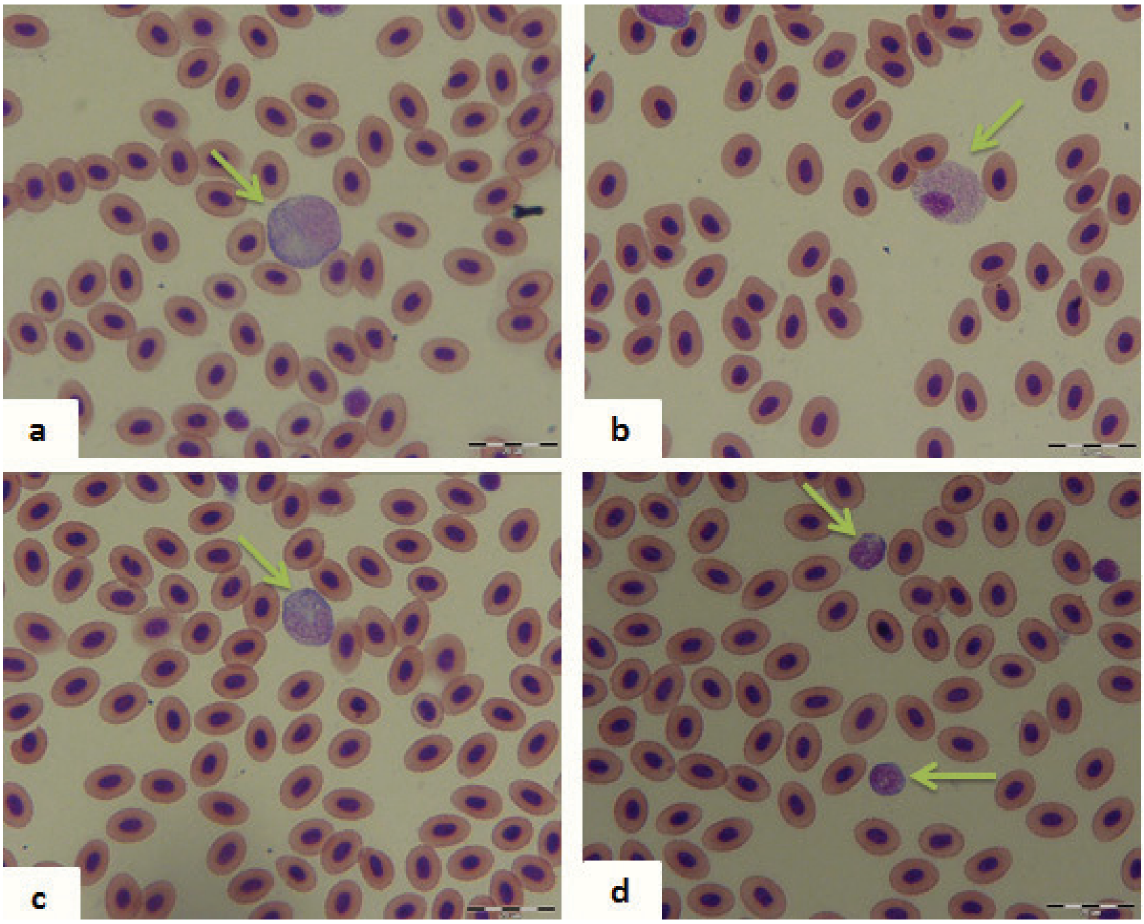

Figure 1. Types of leukocytes (green arrow) in common carp (*Cyprinus carpio*): a – monocyte; b – pseudoeosinophile; c – neutrophil; d – lymphocytes (magnification bar 20 µm)

Table 3. Hematological parameters of the experimental group of common carp

Hematological parameters		WBC $x10^9/l$	Seg	Nonseg	Ne	Ly	Mo	Pse
Total	Mean	7.66	2.81	8,71	10.58	50.92	2.87	35.83
	Standard deviation	2.57	2.37	3.59	4.38	8.28	1.22	9.52
	Minimum	3.70	1.00	3.00	3.00	33.00	1.00	14.00
	Maximum	10.00	16.00	20.00	67.00	5.00	60.00	60.00
	Coefficient of variation - %	33.51	84.36	41.26	41.41	16.27	42.43	26.56
Male	Mean	6.58	2.38	10.00	11.73	49.45	3.18	35.64
	Standard deviation	3.04	1.77	2.65	2.94	5.73	1.25	6.23
	Minimum	3.70	1.00	6.00	8.00	40.00	1.00	25.00
	Maximum	11.70	6.00	14.00	16.00	60.00	5.00	44.00
	Coefficient of variation - %	46.28	74.43	26.46	25.03	11.59	39.30	17.49
Female	Mean	8.58	3.25	7.62	9.62	52.15	2.58	36.00
	Standard deviation	1.71	2.92	4.01	5.24	10.02	1.16	11.88
	Minimum	5.90	1.00	3.00	3.00	33.00	1.00	14.00
	Maximum	10.65	10.00	16.00	20.00	67.00	4.00	60.00
	Coefficient of variation - %	19.92	89.71	52.67	54.46	19.22	45.08	33.00

Table 4. Gender specific analysis of mean values of common carp

Parameter	WBC x 10^9/l	Seg	Nonseg	Ne	Ly	Mo	Pse
			Total				
Control group	5.30	3.30	28.64	31.68	52.12	2.41	13.40
Experimental group	7.66	2.81	8.71	10.58	50.92	2.87	35.83
p-values	0.00.	n.s.	0.00.	0.00.	n.s.	n.s.	0.00.
			Male				
Control group	5.24	3.09	30.00	33.09	55.82	2.20	8.91
Experimental group	6.58	2.38	10.00	11.73	49.45	3.18	35.64
p-values	n.s.	n.s.	0.00.	0.00.	0.01	0.03	0.00.
			Female				
Control group	5.34	3.50	27.57	30.57	49.21	2.57	16.93
Experimental group	8.58	3.25	7.62	9.62	52.15	2.58	36.00
p-values	0.00.	n.s.	0.00.	0.00.	n.s.	n.s.	0.00.

Seg, Nonseg and Mon, except for the value of Ly and Pse. Larger variation of hematological parameters were noticed during the thermal stress. The largest variations were among the segmented neutrophils (CV=89.71% for females). The analyzed parameters of males showed moderate distribution in relation to females, except of WBC and Ly number. Gender specific analysis of mean values between control and experimental group is shown in Table 4.

Comparing the mean values of the control and experimental fish, there was an increase in the number of white blood cells, Mo and Pse, while Seg, Nonseg and Ly were decreased. Statistically significant differences (p=0.00.) were found for the WBC, Nonseg and Pse between control and experimental fish. Experimental group of males showed increased number of WBC, Mo and Pse, where significant differences were found for Nonseg, Ne, Pse (p=0.00.), Ly (p=0.01) and Mo (p=0.03). In experimental females, increase in total WBC number, Ly, Mo and Pse was evident, while significant differences (p=0.00.) were obtained for the WBC, Nonseg, Ne and Pse.

DISCUSSION

Adaptation mechanisms to water temperature are very important and they can be monitored by hematological analysis. To our knowledge, there is no available data for hematological parameters in similar conditions for the common carp, which makes our study even more important. The importance of this study is in the fact that Pse are one of the most present form of leukocytes in the common carp's blood. It is considered that these types of cells can take the roles of other leukocytes and the function of cell is determined by a dominant type of granules (24). This research was confirmed by Hasković et al. (21). The hematopoiesis of that type of cell happens faster and only one type of cell proliferates. Our study showed that they were present in the blood and their number was increased during thermal stress, but they did not become the dominant form of leukocytes.

In the peripheral circulation of fish exposed to elevated water temperatures, an increased number of Pse can be observed as a result of catecholamines and cortisol effects, whose secretion was stimulated by thermal stress (25). Cortisol and catecholamines accelerate the maturation of granulocytes in hematopoietic centers, especially Pse, which were obviously the first hematological cells that enter into the circulatory system as a consequence of any stress. Interrenal chromaffin tissue produces a number of interleukins (IL3, IL3R, IL5) (25) colony stimulating factors and growth factors which stimulate the maturation and ejection of Pse in peripheral circulation. Previous studies on Tilapia and tench (22, 26) showed that Pse and Ly were dominant cells (more than 65%) in the blood which is consistent with our research.

In the case of monocytes, complex fish proinflammatory cytokines triggered mobilization (27, 28) and they cross from pronephros melanomacrophage centers to peripheral circulation (29). Decreased Ly count was probably result of the fact that these cells are involved in the cellular and humoral immune response and could not easily leave the anterior part of pronephros in which they were mainly localized, in relation to Pse and Mo. Ne count was also reduced, suggesting that this type of granulocytes, was not the first line of circulatory and hematological defense of common carp to thermal stress.

Total WBC of experimental males was very similar to WBC of control males, but the proportion of different leukocyte types was changed. This was not the case with the females, where the total number of leukocytes between the control and experimental group was significantly increased, accompanied also with a changed ratio of different types of leukocytes. Females were much more susceptible to thermal stress in comparison to males, because in addition to Pse increase, a significant increase ($p<0.05$) was observed for WBC, which was not the case in comparison to the gender-specific response to thermal stress in previous research on tench (21).

CONCLUSION

Based on the collected data, it is possible to draw the following conclusions: eosinophilic granulocytes, basophilic granulocytes and monocytes in common carp's blood were sporadically present, which is consistent with literature data for tench (21). Short-term hyperthermia lead to an increase of leukocytes number, especially Pse ($p<0.05$) and Mo, while Ly, Seg and Nonseg ($p>0.05$) were reduced. Adaptation mechanism in common carp, caused by higher water temperature, manifests mostly with an increased number of Pse and a decreased number of Ne, though a more detailed explanation of this phenomenon we have not found in the literature. Hematological adaptation of fish to thermal stress is specific for each fish species and there is no uniform hematological response to thermal stress, even among cyprinids, because some cyprinid fish lack Pse in circulation, while the other have large number of Pse, basophils or eosinophils which sheds a completely different light on their thermal adaptation (17, 26).

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