

The effects of rearing conditions on hematology and susceptibility of common carp to experimental manipulation stress

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Abstract: *The effects of rearing conditions on hematology and susceptibility of common carp to experimental manipulation stress.* The aim of present study was to compare hematological values in pond-reared and hatchery-reared common carp, and their hematological alterations following experimental manipulation. Two groups of carp: pond, and hatchery-reared juveniles were subjected to experimental manipulation – transfer of each fish for three hours to separate, aerated but confining aquaria. Blood was sampled and hematological parameters were evaluated (erythrocyte and leukocyte counts, hemoglobin concentration, hematocrit, metabolic activity of phagocytes), and differential erythrocyte and leukocyte counts were calculated in the smears. Prior to stress, pond fish showed higher erythrocyte count and hemoglobin concentration, lower erythrocyte volume, and higher leukocyte count and phagocyte activity comparing to the hatchery fish. Reaction of both groups of fish to manipulation also differed. Pond fish showed erythrocyte swelling, and strong leukopenia (lymphopenia and neutropenia), and a decrease in phagocyte activity, while in hatchery fish increase in erythrocyte count and phagocyte activity took place. Rearing conditions significantly affected hematological parameters of fish, pond-reared carp showing higher oxygen transport and immunological capacities comparing to the hatchery-reared ones. Pond fish showed also higher susceptibility to stress.

Key words: fish, stress, blood, erythrocytes, leukocytes

INTRODUCTION

Manipulation related to management or experimental procedures is a source of stress for fish, and thus may alter many physiological parameters, affecting rearing success or research results. Under experimental conditions, some degree of manipulation is involved in every procedure. Harvesting, handling, sorting, holding, and transport are routine practices that can have significant effects on fish physiology and survival (Benfey and Biron 2000, Portz et al. 2006, Barcellos et al. 2011, Schreck et al. 2016).

Stress responses in teleost fishes are manifested as primary, secondary, and in some cases tertiary reactions (Barton 2002). The primary response to stress involves the activation of two major systems: the hypothalamic-pituitary-interrenal (HPI) axis and the sympathetico-chromaffin (SC) system. Stimulation of the HPI axis results in increased circulating levels of cortisol while stimulation of the SC system results in increased circulating levels of adrenaline (Frish and Anderson 2000). The primary stress

response triggers sequential secondary responses that are manifested as changes in various biochemical, physiological, hematological and immunological parameters (e.g. increase in plasma glucose, hematocrit, lactate, heart rate, gill blood flow, potassium, liver glycogen, muscle protein (Barton 2002, Schreck et al. 2016). Secondary responses generally appear within a few minutes to an hour (Portz et al. 2006). If the stress is severe or prolonged, tertiary responses follow. These include reduced growth rate and metabolic scope for activity, decreased disease resistance and reproductive capacity, as well as altered behavior (Schreck et al. 2016).

Fish for experiments may be obtained from various sources – natural waters, rearing ponds, or from a hatchery. Fish transferred from natural reservoirs or ponds are usually allowed to recover from harvest and transport stress, and to acclimate for 2–4 weeks to the laboratory conditions. However, there are no data showing how previous rearing conditions affect susceptibility of fish to manipulation during experimental procedures themselves. Hematological parameters are commonly used as indicators of physiological status of fish, and indicators of various environmental impacts on fish organism (Fazio 2019).

The aim of present study was to evaluate the effects of rearing conditions on hematological parameters of fish, and on their susceptibility to manipulation stress.

MATERIALS AND METHODS

Two groups of juvenile common carp were used in the experiment: both hatched in June in the hatchery of Inland Fisher-

ies Institute in Żabieniec. One group was then reared in earthen pond under extensive conditions (natural food + grain, natural thermal and oxygen conditions), and showed final body mass 42.1 ± 10.2 g. Another group which was all the time reared in the hatchery tank (fed Chironomid larvae + carp starter feed, at constant temperature $22 \pm 0.5^\circ\text{C}$, 60–80% DO saturation level), and reached body mass of 9.7 ± 4.1 g. Both groups of fish were harvested in October, transported (two hours in plastic bags with pond or tank water supplied with pure oxygen), and allowed to acclimate to the laboratory tanks for a month at $22 \pm 0.5^\circ\text{C}$, at 60–80% DO saturation, pH 6.8–7.2, and N-NH_4^+ level 0.3–5.2 mg/l. Water quality parameters were monitored once a week using pH meter (PRL TN 5123, Elwro, Poland), DO meter HI 9143 (Hanna Instruments, USA), and colorimetric kits Visocolor nitrite and Visocolor ammonia (Macherey-Nagel, Germany). Every day, about three hours post feeding 3/4 of water was gently siphoned out so as not to disturb fish and immediately replaced with fresh tap water. The fish were fed once a day carp starter and frozen Chironomid larvae to satiation.

After this time, the fish were divided into 4 groups (10–16 fish in each – numbers of individuals given in Table 1): PC (pond-controls), PS (pond-stressed), HC (hatchery-controls), and HS (hatchery-stressed). Manipulation included transfer of each fish to separate small aerated aquaria for three hours and simulated routine experimental procedure. Size of aquaria was adjusted to fish body length to create confinement conditions: 5 l for pond fish and 2.3 l for hatchery fish. Control fish for blood sampling were

TABLE 1. Changes in hematological parameters in pond-reared and hatchery-reared carps before (PC, HC) and after (PS, HS) experimental manipulation

Parameter	Experimental groups			
	PC (N = 10)	PS (N = 10)	HC (N = 16)	HS (N = 13)
RBC [10^6 mm^{-3}]	2.04 \pm 0.32 ^a	1.90 \pm 0.22 ^a	1.67 \pm 0.25 ^b	1.92 \pm 0.33 ^a
Hb [g dm^{-3}]	80.8 \pm 36.4 ^a	107.4 \pm 28.1 ^b	77.7 \pm 11.8 ^a	74.7 \pm 15.4 ^a
Ht [%]	23.8 \pm 3.7 ^a	27.7 \pm 2.9 ^b	28.3 \pm 1.2 ^b	28.9 \pm 3.5 ^b
Perimeter area [μm^2]	76.8 \pm 4.1 ^a	90.3 \pm 8.7 ^b	85.6 \pm 3.5 ^c	85.2 \pm 2.9 ^c
MCV [fl]	118 \pm 18 ^a	148 \pm 25 ^b	173 \pm 24 ^c	153 \pm 20 ^b
MCH [pg]	32.9 \pm 16.3 ^a	56.9 \pm 14.6 ^b	47.2 \pm 8.0 ^b	39.6 \pm 9.0 ^a
MCHC[g dm^{-3}]	282 \pm 118 ^a	387 \pm 89 ^b	270 \pm 36 ^a	260 \pm 54 ^a
Erythroblasts [%]	3.1 \pm 1.4 ^a	0.9 \pm 0.5 ^b	3.1 \pm 5.5 ^a	5.5 \pm 3.2 ^c
Abnormal erythrocytes [%]	3.6 \pm 0.9 ^a	4.7 \pm 1.2 ^b	2.2 \pm 0.7 ^c	1.9 \pm 0.8 ^c
Amitotic erythrocytes [%]	0.2 \pm 0.2 ^a	0.6 \pm 0.5 ^b	0.3 \pm 0.2 ^a	1.5 \pm 1.4 ^b
Hemolysed erythrocytes [%]	0.7 \pm 0.6 ^a	1.5 \pm 0.8 ^b	1.1 \pm 0.7 ^a	2.2 \pm 1.3 ^b
WBC [10^3 mm^{-3}]	93.3 \pm 3.7 ^a	64.7 \pm 24.9 ^b	33.5 \pm 11.3 ^c	36.7 \pm 15.7 ^c
Lymphocytes [%]	91.8 \pm 5.9 ^a	92.1 \pm 3.8 ^a	91.7 \pm 3.7 ^a	88.2 \pm 8.5 ^a
Small [%]	86.2 \pm 7.3 ^a	75.5 \pm 13.5 ^b	88.3 \pm 6.4 ^{ab}	76.6 \pm 11.2 ^b
Large [%]	5.6 \pm 3.2 ^a	16.6 \pm 12.9 ^b	8.3 \pm 5.8 ^{ac}	11.5 \pm 5.3 ^{bc}
Lymphocytes [10^3 mm^{-3}]	85.8 \pm 8.3 ^a	59.9 \pm 23.9 ^b	30.8 \pm 10.9 ^b	32.7 \pm 15.4 ^b
Neutrophils [%]	5.8 \pm 4.4 ^{ab}	2.6 \pm 1.8 ^a	6.6 \pm 3.4 ^b	10.7 \pm 7.8 ^b
Myelocytes [%]	3.6 \pm 2.6 ^a	0.6 \pm 0.7 ^b	1.8 \pm 1.6 ^{bc}	2.7 \pm 2.1 ^{ac}
Metamyelocytes [%]	1.3 \pm 1.5	0.7 \pm 0.5	1.9 \pm 1.8	2.1 \pm 2.2
Band [%]	0.2 \pm 0.5 ^a	0.4 \pm 0.7 ^a	1.3 \pm 1.1 ^b	2.7 \pm 2.4 ^b
Segmented [%]	0.7 \pm 1.5 ^a	0.9 \pm 1.7 ^a	1.4 \pm 1.3 ^{ab}	3.3 \pm 2.9 ^b
Neutrophils [10^3 mm^{-3}]	5.3 \pm 3.8 ^a	1.6 \pm 1.2 ^b	2.1 \pm 1.2 ^b	3.6 \pm 2.4 ^{ab}
NBT [g dm^{-3} of formasan]	1.5 \pm 0.7 ^a	0.9 \pm 0.4 ^{bc}	0.6 \pm 0.1 ^b	0.8 \pm 0.1 ^c
Monocytes [%]	1.9 \pm 1.6 ^{ab}	3.5 \pm 2.1 ^a	1.6 \pm 1.1 ^b	0.7 \pm 1.0 ^c
Eosinophils [%]	0.3 \pm 0.5	0.3 \pm 0.5	0.3 \pm 0.5	0.2 \pm 0.4
Basophils [%]	0.2 \pm 0.4	1.5 \pm 2.8	0.2 \pm 0.4	0.3 \pm 0.5
Thrombocytes [10^3 mm^{-3}]	20.8 \pm 11.3	20.7 \pm 17.5	13.0 \pm 12.4	19.6 \pm 19.2

significant differences marked with different letter superscripts, $P < 0.05$, U-test, $a \neq b \neq c$.

harvested directly from acclimation tanks. After this time, blood was collected from live fish by heart puncture using chilled heparinized needles into chilled heparinized Eppendorf tubes. Blood from each fish was sampled only once.

Fresh blood was subjected to hematological analyses. Hematocrit value (Ht) was evaluated after blood centrifugation in microhematocrit centrifuge at 12,000 rpm for 5 min and the results were obtained using microhematocrit reader. Red and white blood cell counts (RBC, WBC) were calculated in Burkner chamber, at 100 times dilution with Hayem liquid. Hemoglobin concentration (Hb) was measured using cyanmethemoglobin spectrophotometric method (540 nm) with Drabkin solution. The derived red blood parameters were also counted MCV (mean cell volume) as $Ht \times 10 \times RBC^{-1}$, MCH (mean cell hemoglobin content) as $Hb \times RBC^{-1}$, and MCHC (mean cell hemoglobin concentration) as $Hb \times 100 \times Ht^{-1}$. The spontaneous oxidative metabolic activity of phagocytes (as NBT reduction to formazan) was also measured in fresh blood using spectrophotometric method (at 546 nm). Blood smears were made, air dried, and stained using May-Grunwald and Giemsa solutions. In each smear 300 erythrocytes, 100 leucocytes, and number of thrombocytes accompanying 100 leucocytes were counted. Evaluation of erythrocytes included measurement of perimeter area (20 cells in each smear) and calculation of percentages of erythroblast and various types of abnormal cells. Differential leukocyte count was also calculated and thrombocyte count was estimated based on number of thrombocytes per 100 leucocytes in smear and WBC value. The

smears were viewed using light microscope (1000 × magnification). The results were subjected to statistical analysis (Mann-Whitney U-test, $P < 0.05$) to evaluate the significance of differences.

RESULTS

The obtained results show that rearing conditions affected blood composition of fish. Pond-reared fish (PC) had higher RBC, lower Ht, MCH and smaller erythrocyte size comparing to HC, while the difference in Hb concentration was insignificant due to high variability in PC group. They showed also significantly higher WBC, and absolute lymphocyte and neutrophil counts. The average number of neutrophils was over twice higher in the PC, and so was the phagocyte respiratory activity (NBT). Percentage of various types of white blood cells did not very much differ between the groups – the only difference was higher percentage of juvenile neutrophils (metamyelocytes) in the PC group, while the HC fish had more mature (band and segmented) cells.

Pond and hatchery fish reacted to experimental manipulation in a different way. Most erythrocyte parameters: Hb, Ht, MCV (and directly measured erythrocyte perimeter area), MCHC, and MCH significantly increased in the PS group, comparing to PC. At the same time, a significant decrease in erythroblast contribution, and an increase in frequency of erythrocyte cellular abnormalities, and hemolysis occurred. Deep alterations were also observed in leukocyte system of these fish – a considerable decrease in WBC, accompanied by increased in-

dividual variability, a significant shift from small to large lymphocytes, and a decrease in lymphocyte and neutrophil counts accompanied by a substantial drop in phagocyte respiratory activity.

In the hatchery-reared fish RBC significantly increased, while MCV and MCH decreased. This was accompanied by an increase in erythroblast percentage, as well as the frequency of amitotic (dividing), and hemolysed erythrocytes. On the other hand, no significant changes in leukocyte parameters were observed in the hatchery fish, except for an increase in phagocyte respiratory burst, and monocyte percentage.

Thrombocyte count was highly variable, and did not show significant differences.

DISCUSSION

Fish show considerable intraspecific differences of hematological values that depend on various intrinsic and environmental factors (Thomas et al. 1999, Fazio 2019). Rearing conditions applied prior to manipulation significantly affected hematological parameters of fish. Higher number of smaller erythrocytes in the pond fish comparing to the hatchery fish might have been related to the more variable oxygen and water temperature levels experienced by the fish reared under natural conditions comparing to the stable conditions in the hatchery tanks. A functional basis is found in larger surface area to volume ratios and shorter diffusion distances allowing more rapid oxygenation and deoxygenation of hemoglobin as erythrocyte volume decreases (Lay and Baldwin 1999). The results

reported by Rehulka and Adamec (2004) have shown that fish farming technology, varying physical and chemical properties of water and availability of natural food may increase erythropoiesis (higher Ht, Hb, RBC, MCV, MCH, MCHC in cage comparing to tank fish).

Higher WBC, and phagocyte metabolic activity (NBT) in the pond fish was probably related to their natural exposure to pathogens, and better development of immune system, comparing to the hatchery fish reared in clean water, at the absence of any pathogenic microorganisms.

It is generally agreed that many different types of stress result in changes in blood cell numbers and activities. Increase in red blood cell parameters is an adaptive reaction of stressed fish enhancing oxygen carrying capacity of blood, and enabling the organism higher energy production (Eslamloo et al. 2014). Under stress conditions, increase in Ht, Hb, red cell count and volume usually take place (Houston et al. 1996, Suski et al. 2007, Olsen et al. 2008). Both groups of carp subjected to manipulation showed alterations in blood parameters but in each group the changes were different. PS fish showed symptoms of erythrocyte swelling, while in HS fish erythrocyte count and contribution of erythroblasts increased.

Stress-induced swelling of fish erythrocytes is a result of adrenergic osmotic alterations (Na^+ and K^+ transport into the cells, and subsequent water uptake), accompanied by an acidification of plasma, and alkalization of erythrocyte cytoplasm (Nikinmaa and Huestis 1984). Diluted hemoglobin under alkaline conditions shows higher oxygen affinity, thus eryth-

rocyte swelling is an adaptive reaction that results in a low-cost increase of oxygen transport efficiency (Kind et al. 2002). Significant increase in Hb without an elevation of RBC is, however, somewhat unclear. According to Speckner et al. (1989), fish erythrocytes can synthesize hemoglobin while circulating in peripheral blood but no data are available on the rate of this process.

Rapid increase in erythrocyte number (causing even 25% increase in Ht) is a result of spleen contraction (Barcellos et al. 2004) and 90% of new cells may be released within several minutes (Houston et al. 1996). In both groups of stressed fish the frequency of bilobed (amitotic) erythrocytes increased. Sometimes division of circulating cells may be observed (especially in case of hypoxia stress) (Murad et al. 1993), and this mechanism may be considered probably another adaptive reaction increasing the number of cells. The increase in percentage of abnormal erythrocytes in PS, and of hemolysed cells in both groups subjected to stress might have been related to increased membrane fragility in swollen cells, or osmotic alterations.

An increase in Ht and Hb without a change in RBC was reported by Benfey and Biron (2000) for *Oncorhynchus mykiss* and *Salvelinus fontinalis* subjected to acute handling or confinement stress. A stress-related increase in Ht and Hb was also observed by Frish and Anderson (2000) in *Plectropomus leopardus*. Increase in Ht and MCV values, and a decrease in MCHC value during harvest have been also observed by Svobodova et al. (2006).

Considerable differences between pond and hatchery fish occurred in the re-

action of leukocyte parameters to stress. In the PS group WBC (both lymphocytes and neutrophils), and phagocyte activity (NBT) significantly decreased, while in HS no WBC change took place, and NBT significantly increased.

Fish immune responses to stress indicate that the type, intensity, and duration of the stressor are major determinants of the effect of stress on immune functions (Portz et al. 2006). An important secondary effect of stress in fish is immunosuppression (Ortuno et al. 2001). Cortisol levels increase in the initial stages of stressful situations (Svobodova et al. 1999, Barcellos et al. 2011). It is known that cortisol secreted during stress reaction shortens the life span of lymphocytes and promotes their apoptosis (Verburg van Kemenade et al. 1999), and reduces their proliferation (Espelid et al. 1996), so a decrease in the number of lymphocytes in the circulating blood, as well as in their activity are often observed effects of stress, irrespectively of the stressing agent. The reduction in the number of circulating lymphocytes in stressed fish may partly be due to the extravasation of those cells and their penetration to the epithelium of the gills, skin or the intestine (Svobodova et al. 2006). Benfey and Biron (2000) observed that acute stress was followed by marked leukopenia in *Oncorhynchus mykiss*, and *Salvelinus fontinalis*, due specifically to lymphopenia. The decrease in the concentration of lymphocytes and granulocytes observed Frish and Anderson (2000). Lymphopenia and neutrophilia in carp subjected to stress induced by harvest and post-harvest storage were observed by Svobodova et al. (2006). According to Ortuno et al. (2001), monocytes and granulocytes

are mobilized to the circulating blood from the head kidney for 48 h after exposure to a stressor. Despite their significant increase in number, the phagocytic ability of these leukocytes is usually significantly reduced immediately after stress (apparently due to incomplete development), and normal immunocompetence may not recover for more than 48 hours (Ortuno et al. 2001, Vazzana et al. 2002). In the present study, no neutrophilia or monocytosis was observed, on the contrary – PS fish showed neutropenia, and reduction in phagocyte activity. Phagocyte respiratory burst was reduced in *Dicentrarchus labrax* subjected to confinement (Vazzana et al. 2002), and *Danio rerio* after high and low temperature stress (Mustafa et al. 2008).

CONCLUSIONS

The obtained results show that mild manipulation resulted in a stress reaction in both groups of fish but the strength of the response was different. In both groups red blood parameters showed an adaptive increase (more pronounced in the pond fish group). The reaction of white blood cell system was, however, completely different – strong symptoms of immunosuppression occurred in PS group, and an enhancement of phagocyte activity in the HS group. These results indicate that the fish obtained from ponds were more susceptible to manipulation stress comparing to those reared for all their life in hatchery tank, and that the usually applied period of 2–4 weeks of acclimation to the laboratory conditions may be sufficient to recover from harvest & transport stress.

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Streszczenie: *Wpływ warunków hodowli na parametry hematologiczne i podatność karpia na eksperymentalny stres manipulacyjny.* Celem prezentowanej pracy było porównanie wartości hematologicznych u karpia hodowanych w stawie i wylęgarni oraz zmian hematologicznych po eksperymentalnych manipulacjach. Dwie grupy karpia: wyhodowane w stawie oraz w wylęgarni poddano manipulacjom doświadczalnym – w tym celu przeniesiono każdą rybę na trzy godziny do oddzielnego, napowietrzanego, ale zamkniętego akwarium. Pobrano próbki krwi i oceniono parametry hematologiczne (liczba erytrocytów i leukocytów, stężenie hemoglobiny, hematokryt, aktywność metaboliczna fagocytów) oraz obliczono udział poszczególnych grup erytrocytów i leukocytów na rozmazach. U ryb stawowych przed poddaniem stresowi stwierdzono większą liczbę erytrocytów i stężenie hemoglobiny, mniejszą objętość erytrocytów oraz większą liczbę leukocytów i aktywność fagocytów, w porównaniu z rybami z wylęgarni. Reakcja obu grup

ryb na manipulację również się różniła. We krwi ryb stawowych odnotowano pęczniecie erytrocytów i silną leukopenię (limfopenię i neutropenię) oraz spadek aktywności fagocytów, natomiast u osobników z wylęgarni nastąpił wzrost liczby erytrocytów i aktywności fagocytów. Warunki hodowlane istotnie wpłynęły na parametry hematologiczne ryb, karpie stawowe charakteryzowała wyższa liczba krwinek czerwonych (co umożliwiło lepszy transport tlenu) oraz większa odporność immunologiczna (wynikająca ze zwiększenia metabolicznej aktywności fagocytarnej krwinek) w porównaniu do ryb pochodzących z hodowli w wylęgarni. Ryby stawowe wykazywały większą podatność na stres.

Słowa kluczowe: ryby, stres, krew, erytrocyty, leukocyty

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