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The effects of heparin concentration, storage time, and temperature on the values of hematological parameters in *Cyprinus carpio*

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Abstract: The results of hematological analyses may be affected by preanalytical factors such as sampling procedure, type and concentration of anticoagulant, or conditions and time of blood storage. In the present study the effects of various concentrations of heparin (0–200 IU/cm^3) and different times (2, 24, or 48 h) and temperatures (4 or 22 °C) of blood storage on the values of hematological parameters in *Cyprinus carpio* were evaluated. Increase in heparin concentration resulted in a decrease in frequency of erythroblasts, a reduction of leukocyte and thrombocyte counts, and a decrease in oxidative metabolic activity of phagocytes. Storage for 2 h at 22 °C resulted in a decrease in erythrocyte, leukocyte, and thrombocyte counts, while at 4 °C an increase in corpuscular hemoglobin values and a beginning of decrease in leukocyte count occurred after 24 h. These results indicate that heparin concentrations should be minimized and equal for all blood samples (preferably 50 IU/cm^3), blood must be refrigerated immediately after sampling, and the analyses should be performed not later than 1 day after sampling.

Key words: Anticoagulant, blood storage, carp, fish, hematology, heparin

1. Introduction

Hematological parameters are important indicators of fish physiological status (1) and their values are sensitive to environmental changes (2–4). Blood sampling is easy and in most cases harmless to fish. Small amounts of collected blood may be used to measure many various parameters that provide abundant information about the organism. However, the usefulness of blood for analyses and thus the reliability of the obtained results depend on sampling, processing, and storage procedures, including the level of stress, use of anesthetics, type and concentration of anticoagulant, storage temperature, and storage time (5–7). Fish blood easily and quickly coagulates (8,9), and therefore the use of anticoagulants is usually necessary (10). According to Mainwaring and Rowley (6), Korcock et al. (8), and Walencik and Witeska (11), heparin is the most appropriate anticoagulant for analysis of fish blood. According to Svobodova et al. (10), a heparin concentration of 50 IU/cm^3 is recommended but a slight overdose does not affect the results. On the other hand, Mainwaring and Rowley (6) reported occasional small cell clumps in blood at 50 IU/cm^3 of heparin, which indicates that sometimes higher doses of anticoagulant may be necessary. Our observations showed that disease may accelerate blood coagulation in fish, and in such a case an increase in heparin concentration to 100 IU/cm^3 or more is necessary to prevent clotting. This may be a stress-related symptom since stress considerably accelerates blood coagulation (12,13). However, there are very little data concerning the effects of various concentrations of heparin on the values of hematological parameters in fish. According to Smit et al. (14), a slight decrease in hematocrit occurred in blood with the increase in heparin concentration from 0.5 to 8.0 mg/cm^3. Mainwaring and Rowley (6) reported reduction of leukocyte viability at higher concentrations of heparin (600–3000 IU/cm^3). On the other hand, Teixeira de Oliveira et al. (15) reported no differences in red blood parameters between samples treated with 2500 and 5000 IU/cm^3 of heparin. Similarly, Vaz Farias et al. (16) found no differences in the values of hematological parameters between samples with 100 and 5000 IU/cm^3 of heparin.

Sometimes (e.g., in the case of sampling in field) hematological analyses cannot be performed immediately after blood collection and samples must be stored for some time. The results of various studies indicate that storage conditions and time may affect the values of hematological parameters. Storage of blood for less than 24 h was recommended by Korcock et al. (8) and Faggio et al. (17), while according to Faggio et al. (1), most hematological parameters should be assessed even within

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6 h after sampling. Very little is known about the effect of temperature on the usefulness of fish blood for analyses. According to Tavares-Dias and Silva Sandrim (18), 10 h of storage at room temperature did not significantly affect the values of hematological parameters.

The present study was undertaken to evaluate the effects of various concentrations of heparin, storage temperature, and time on the values of blood parameters in *Cyprinus carpio*.

2. Materials and methods

Three experiments were performed: experiments 1 and 2 to investigate the effects of various heparin concentrations, and experiment 3 to evaluate the influence of blood storage time and temperature on the values of hematological parameters of common carp.

Blood was collected from healthy juvenile common carp (*Cyprinus carpio*) individuals of body mass 122 ± 31 g. The fish, obtained from the ponds of the Inland Fisheries Institute in Żabieniec, were kept for 12 months in an aerated flow-through tank of 300 dm^3 at a temperature 18.0 ± 1.0 °C and dissolved oxygen saturation of 70%–80%. The fish were fed daily in the morning to satiation with carp feed Aller Aqua Classic 4 mm.

For evaluation of the effects of heparin concentration on hematological parameters, 500–600 mm^3 of blood was collected from the fish using heparinized chilled needles into nonheparinized chilled plastic tubes. The attempts of blood sampling with nonheparinized needles resulted in immediate blood coagulation. The fish were individually netted from the rearing tank and handled in a wet cloth so that their eyes were covered. Blood was sampled by heart puncture and the entire procedure from the harvest to returning the fish back to the tank lasted no more than 30 s. Immediately after collection, 100 mm^3 of each sample was transferred into Eppendorf tubes containing 0, 10, 50, or 100 IU (experiment 1, n = 20) or 0, 10, 25, 50, 100, or 200 IU (experiment 2, n = 17) of heparin per 1 cm^3 of blood and incubated for 2 h at 4 °C. The solutions were made of heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich).

For evaluation of the effects of storage time and temperature, 500 mm^3 of blood was collected (experiment 3, n = 10) using chilled heparinized needles into chilled plastic tubes containing 50 IU/cm^3 of heparin. The blood of each fish was placed in 2 Eppendorf tubes (200 mm^3 in each). One set of tubes was stored in the refrigerator at 4 °C and another was left at room temperature (22 °C). Subsamples of blood were analyzed after 2, 24, and 48 h. The samples kept at 22 °C coagulated after 48 h and analyses were no longer possible.

Blood samples were subjected to hematological analysis according to Svobodova et al. (10). Hematocrit (Ht), hemoglobin concentration (Hb), erythrocyte count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leukocyte count (WBC), and oxidative metabolic activity of phagocytes (nitro tetrazolium blue test, NBT) were evaluated. Blood smears were also made and stained with May–Grünwald and Giemsa solutions for differential erythrocyte and leukocyte counts, for estimation of thrombocyte count (TC), and for differential thrombocyte count (only in experiment 2).

For Ht evaluation heparinized capillaries with blood were centrifuged at 12,000 rpm for 5 min and then the percentage of the erythrocyte layer in the entire blood volume was measured using the Ht reader. Hb was measured using the spectrophotometric cyanmethemoglobin method: 10 mm^3 of blood was mixed with 1 cm^3 of Drabkin solution, extinction was read at the 540 nm wavelength, and hemoglobin concentration was calculated from the equation of the relationship between the extinction and concentrations of standard hemoglobin solutions. For RBC and WBC counts blood was diluted 100 times with Hayem solution and the cells were counted in a Burker hemocytometer under 400× magnification. The values of MCV, MCH, and MCHC were calculated using Ht, RBC, and Hb values according to the following formulas:

MCV = (Ht × 10) / RBC, MCH = Hb / RBC, and MCHC = (Hb × 100) / Ht. Oxidative metabolic activity of phagocytes was measured according to Studnicka et al. (19) using the NBT reduction test: 50 mm^3 of blood was measured with an equal amount of 0.2% NBT solution and incubated for 1 h at 28 °C (mixed every 15 min), and then 1 cm^3 of dimethylformamide (DMF) was added to kill the cells and the samples were shaken for 5 min to disrupt cell membranes and liberate formazan (the product of NBT reduction). Extinction was read at the 546 nm wavelength using the spectrophotometer and formazan concentration was calculated from the equation of the relationship between the extinction and concentrations of standard formazan solutions. TC was estimated from the number of thrombocytes per 100 leukocytes in a blood smear and WBC values. Differential erythrocyte, leukocyte, and thrombocyte counts were done based on the analyses of blood smears (300 erythrocytes, 100 leukocytes, and 100 thrombocytes were viewed and identified in each smear).

The results were subjected to statistical analysis using Statistica software. Prior to the evaluation of significance of differences, the results were subjected to the Shapiro–Wilk test, which showed the nonnormal distribution of most of the variables. Therefore, the significance of differences in the values of hematological parameters among experimental groups of blood samples was

evaluated using the nonparametric Kruskal–Wallis test, assuming a significance level of $P \leq 0.05$.

This project was approved by the III Local Ethical Committee at the Warsaw University of Life Sciences.

3. Results

The results of both experiments showed that heparin concentrations of 0–200 IU/cm^3 did not significantly affect most of the red blood cell parameters in carp (Tables 1 and 2). However, in experiment 1 the frequency of erythroblasts decreased with the increase of anticoagulant level, which did not take place in experiment 2. On the other hand, in experiment 2 a slight but insignificant increase in MCV values occurred at heparin concentrations of 50–200 IU/cm^3. The frequency of erythrocytes showing morphological anomalies ranged from 0.8 ± 0.7% to 1.5 ± 0.9% (experiment 1) and from 1.9 ± 1.0% to 2.6 ± 1.4% (experiment 2) and did not significantly differ among the groups.

In experiment 1 a significant decrease in WBC count was observed at 50 and 100 IU/cm^3 compared to the nonheparinized blood, while in experiment 2 no such effect occurred. No changes in differential leukocyte counts were observed. However, in experiment 2 a significant decrease in the spontaneous oxidative metabolic activity of phagocytes (NBT) was noted at the highest level of anticoagulant (200 IU/cm^3) compared to the nonheparinized blood. In experiment 1 TC values were significantly lower at 50 and 100 IU/cm^3 than in blood without anticoagulant, while in experiment 2 no significant alterations in TC occurred. However, a slight shift towards round thrombocytes and a decrease in frequency of elongated thrombocytes was observed.

The results concerning the effects of storage conditions (Table 3) revealed a slight insignificant decrease in RBC count and an increase in erythroblast and abnormal erythrocyte frequency (from 1.3 ± 0.9% in 2 h at 4 °C to 3.4 ± 2.1% in 24 h at 22 °C), MCV, and MCH with time and temperature increase. WBC and TC values decreased with time and temperature, while the oxidative metabolic activity of phagocytes was significantly reduced only by increased temperature. No alterations were observed in differential leukocyte count.

4. Discussion

The results of both experiments concerning the effects of various heparin concentrations revealed that the values of most hematological parameters remained unaffected by this anticoagulant. The results obtained in both experiments were slightly different: in experiment 1 the percentage of erythroblasts, WBC count, and TC significantly decreased with increase in heparin concentration, while in experiment 2 a significant decrease in the oxidative metabolic activity of phagocyte cells (NBT) was the only significant change observed, accompanied by a slight and insignificant increase in MCV and changes in differential thrombocyte count with an increase in the percentage of round thrombocytes and decrease in the frequency of elongated ones.

It was proved that heparin is the most appropriate anticoagulant for analyses of fish blood (11,16,20). However, in the literature there are very little data concerning the effects of various concentrations of heparin on the values of hematological parameters. Mainwaring and Rowley (6) reported that high concentrations of heparin (600–3000 IU/cm^3) reduced leukocyte viability

Table 1. The effect of heparin concentrations (experiment 1) on the values of hematological parameters of common carp (different letters as superscripts indicate significant differences; Kruskal–Wallis test, n = 20, $P \leq 0.05$).

Parameter	0 IU/cm^3	10 IU/cm^3	50 IU/cm^3	100 IU/cm^3
RBC [10^6/mm^3]	2.3 ± 0.6 ^{a}	2.3 ± 0.5 ^{a}	2.1 ± 0.5 ^{a}	2.2 ± 0.8 ^{a}
Ht [%]	25.4 ± 4.3 ^{a}	23.8 ± 3.7 ^{a}	23.2 ± 3.9 ^{a}	23.2 ± 3.6 ^{a}
Hb [g/dm^3]	114 ± 30 ^{a}	120 ± 29 ^{a}	121 ± 32 ^{a}	104 ± 31 ^{a}
MCV [fL]	115 ± 24 ^{a}	107 ± 24 ^{a}	116 ± 29 ^{a}	113 ± 32 ^{a}
MCH [pg]	51.4 ± 15.2 ^{a}	53.8 ± 16.4 ^{a}	59.9 ± 20.8 ^{a}	47.9 ± 11.9 ^{a}
MCHC [g/dm^3]	445 ± 80 ^{a}	508 ± 126 ^{a}	525 ± 131 ^{a}	455 ± 134 ^{a}
Erythroblasts [%]	4.4 ± 2.2 ^{a}	2.6 ± 1.4 ^{ab}	2.6 ± 2.0 ^{ab}	1.8 ± 0.9 ^{b}
WBC [10^3/mm^3]	36.8 ± 9.9 ^{a}	28.1 ± 10.4 ^{ab}	26.5 ± 7.3 ^{b}	26.3 ± 8.1 ^{b}
Lymphocytes [%]	96.5 ± 2.8 ^{a}	96.6 ± 1.7 ^{a}	96.5 ± 2.5 ^{a}	95.7 ± 2.5 ^{a}
Neutrophils [%]	2.4 ± 1.8 ^{a}	2.7 ± 1.5 ^{a}	2.4 ± 1.7 ^{a}	3.1 ± 2.1 ^{a}
NBT [g/dm^3 of formazan]	0.4 ± 0.2 ^{a}	0.6 ± 0.3 ^{a}	0.5 ± 0.2 ^{a}	0.5 ± 0.2 ^{a}
TC [10^3/mm^3]	34.8 ± 9.9 ^{a}	26.6 ± 13.2 ^{ab}	22.7 ± 8.2 ^{b}	23.5 ± 8.3 ^{b}

Table 2. The effect of heparin concentrations (experiment 2) on the values of hematological parameters of common carp (different letters as superscripts indicate significant differences; Kruskal–Wallis test, n = 17, P ≤ 0.05).

Parameter	0 IU/cm^3	10 IU/cm^3	25 IU/cm^3	50 IU/cm^3	100 IU/cm^3	200 IU/cm^3
RBC [10^6/mm^3]	1.29 ± 0.26 ^{a}	1.32 ± 0.22 ^{a}	1.32 ± 0.29 ^{a}	1.19 ± 0.18 ^{a}	1.21 ± 0.20 ^{a}	1.26 ± 0.34 ^{a}
Ht [%]	20.2 ± 3.8 ^{a}	21.3 ± 3.3 ^{a}	21.2 ± 3.6 ^{a}	20.9 ± 3.5 ^{a}	21.4 ± 3.5 ^{a}	21.1 ± 3.3 ^{a}
Hb [g/dm^3]	53 ± 17 ^{a}	56 ± 19 ^{a}	60 ± 17 ^{a}	61 ± 14 ^{a}	55 ± 16 ^{a}	54 ± 15 ^{a}
MCV [fL]	162 ± 32 ^{a}	163 ± 22 ^{a}	167 ± 24 ^{a}	184 ± 23 ^{a}	180 ± 26 ^{a}	180 ± 47 ^{a}
MCH [pg]	43.8 ± 17.4 ^{a}	43.9 ± 17.7 ^{a}	47.2 ± 14.1 ^{a}	51.2 ± 10.5 ^{a}	46.1 ± 14.4 ^{a}	45.8 ± 16.1 ^{a}
MCHC [g/dm^3]	267 ± 99 ^{a}	270 ± 99 ^{a}	285 ± 77 ^{a}	281 ± 68 ^{a}	258 ± 88 ^{a}	262 ± 83 ^{a}
Erythroblasts [%]	2.7 ± 1.4 ^{a}	3.5 ± 1.6 ^{a}	3.5 ± 1.4 ^{a}	2.7 ± 0.9 ^{a}	3.5 ± 1.8 ^{a}	3.4 ± 1.8 ^{a}
WBC [10^3/mm^3]	31.6 ± 19.8 ^{a}	29.7 ± 11.4 ^{a}	30.6 ± 10.5 ^{a}	32.7 ± 10.7 ^{a}	35.0 ± 15.4 ^{a}	33.1 ± 17.0 ^{a}
Lymphocytes [%]	95.9 ± 2.8 ^{a}	95.8 ± 3.6 ^{a}	95.9 ± 4.0 ^{a}	97.7 ± 2.5 ^{a}	96.5 ± 4.6 ^{a}	94.9 ± 4.6 ^{a}
Neutrophils [%]	3.1 ± 2.8 ^{a}	2.8 ± 3.2 ^{a}	2.6 ± 2.3 ^{a}	1.9 ± 2.2 ^{a}	2.1 ± 2.7 ^{a}	2.7 ± 2.6 ^{a}
NBT [g/dm^3 of formazan]	0.9 ± 0.4 ^{a}	0.9 ± 0.4 ^{a}	0.9 ± 0.4 ^{a}	0.8 ± 0.4 ^{ab}	0.7 ± 0.3 ^{ab}	0.6 ± 0.2 ^{b}
TC [10^3/mm^3]	12.0 ± 9.0 ^{a}	14.3 ± 8.8 ^{a}	13.9 ± 10.1 ^{a}	16.3 ± 7.5 ^{a}	16.5 ± 9.7 ^{a}	13.6 ± 9.9 ^{a}
Round thrombocytes [%]	62.2 ± 15.8 ^{a}	61.0 ± 20.0 ^{a}	60.5 ± 18.0 ^{a}	61.1 ± 16.5 ^{a}	67.9 ± 14.8 ^{a}	73.6 ± 17.1 ^{a}
Elongated thrombocytes [%]	24.2 ± 12.0 ^{a}	26.4 ± 17.6 ^{a}	27.2 ± 13.6 ^{a}	27.8 ± 19.4 ^{a}	17.3 ± 10.8 ^{a}	13.5 ± 14.5 ^{a}
Spindle thrombocytes [%]	13.6 ± 10.9 ^{a}	12.6 ± 11.4 ^{a}	12.2 ± 12.4 ^{a}	11.1 ± 14.3 ^{a}	14.8 ± 17.7 ^{a}	12.9 ± 14.0 ^{a}

Table 3. The effect of storage time and temperature (experiment 3) on the values of hematological parameters of common carp (different letters as superscripts indicate significant differences; Kruskal–Wallis test, n = 20, P ≤ 0.05).

Parameter	4 °C/2 h	4 °C/24 h	4 °C/48 h	22 °C/2 h	22 °C/24 h
RBC [10^6/mm^3]	1.52 ± 0.23 ^{a}	1.31 ± 0.41 ^{ab}	1.26 ± 0.36 ^{ab}	1.07 ± 0.17 ^{b}	1.26 ± 0.36 ^{ab}
Ht [%]	20.8 ± 3.7 ^{a}	19.4 ± 3.2 ^{a}	19.5 ± 3.1 ^{a}	20.1 ± 4.1 ^{a}	22.2 ± 3.1 ^{a}
Hb [g/dm^3]	46.7 ± 10 ^{a}	56.1 ± 15.2 ^{a}	53.0 ± 17.9 ^{a}	42.9 ± 18.6 ^{a}	54.9 ± 6.9 ^{a}
MCV [fL]	139 ± 29 ^{a}	161 ± 52 ^{a}	159 ± 36 ^{a}	174 ± 41 ^{ab}	206 ± 38 ^{b}
MCH [pg]	31.8 ± 10.4 ^{a}	48.7 ± 21.8 ^{b}	41.3 ± 8.4 ^{a}	37.4 ± 19.3 ^{ab}	51.2 ± 11.3 ^{b}
MCHC [g/dm^3]	228 ± 50 ^{a}	299 ± 64 ^{b}	272 ± 66 ^{ab}	211 ± 76 ^{a}	249 ± 28 ^{ab}
Erythroblasts [%]	4.1 ± 2.6 ^{a}	5.2 ± 1.9 ^{ab}	6.4 ± 2.0 ^{b}	4.8 ± 2.7 ^{ab}	5.9 ± 1.1 ^{ab}
WBC [10^3/mm^3]	54.3 ± 14.5 ^{a}	47.9 ± 15.9 ^{ab}	45.2 ± 14.8 ^{b}	41.0 ± 8.0 ^{b}	42.5 ± 10.1 ^{b}
NBT [g/dm^3 of formazan]	0.72 ± 0.14 ^{a}	0.82 ± 0.20 ^{a}	0.80 ± 0.11 ^{a}	0.65 ± 0.10 ^{ab}	0.37 ± 0.25 ^{b}
Lymphocytes [%]	97.1 ± 2.0 ^{a}	95.9 ± 2.4 ^{a}	96.3 ± 2.2 ^{a}	96.2 ± 2.4 ^{a}	95.6 ± 2.5 ^{a}
Neutrophils [%]	2.1 ± 1.7 ^{a}	2.2 ± 1.3 ^{a}	2.5 ± 2.0 ^{a}	2.9 ± 1.7 ^{a}	3.5 ± 1.9 ^{a}
TC [10^3/mm^3]	43.4 ± 7.9 ^{a}	42.0 ± 12.1 ^{ab}	36.0 ± 8.9 ^{ab}	34.0 ± 6.9 ^{b}	38.3 ± 11.5 ^{ab}

but did not alter differential leukocyte count. Vaz Farias et al. (16) did not observe any significant differences in Ht, Hb, MCHC, frequency of hemolysis, and NBT between blood samples with 100 and 5000 IU/cm^3 of heparin. However, minor increase in MCV and WBC count and a decrease in TC occurred. No differences in the values of red blood parameters between blood samples with 2500 and 5000 IU/cm^3 of heparin were reported by Teixeira de Oliveira et al. (15). These authors reported interesting results concerning blood coagulation: erythrocyte clumps

were present in 40% of samples with lower and in 60% of samples with higher heparin concentrations. Very little data are also available on the effects of heparin on hematological parameters of other vertebrates. According to Nielsen (21), concentrations of this anticoagulant over 20 IU/cm^3 cause a decrease in spontaneous migration and chemotaxis of human phagocytes. On the other hand, Sissener Engstad et al. (22) reported that 10 IU/cm^3 heparin strongly increased monocyte, neutrophil, and thrombocyte cytokine release in human blood. In both experiments no coagulation was observed, even in the nonheparinized tubes; however, blood was always sampled with heparinized needles. These results indicate that blood sampling with minimum anticoagulant use is possible if it is done very quickly and skillfully, and if the fish are in good condition and unstressed.

The obtained results showed that temperature and time of storage affected the values of some parameters: at 4 °C the RBC count gradually but insignificantly decreased, while the percentage of erythroblasts increased. MCH and MCHC significantly increased after 24 h, and after 48 h a decrease in WBC count occurred. At 22 °C RBC and WBC values were lower than at 4 °C after 2 h, and after 24 h the value of NBT significantly decreased, while MCV and MCH increased. After 48 h all the samples coagulated and analyses were not possible. Time- and temperature-related alterations in the values of fish hematological parameters were also reported by other authors. Tavares-Dias and Silva Sandrim (18) found no alterations in Ht, Hb, and MCHC after 10 h at room temperature. Korcock et al. (8) observed an increase in MCV after 24 h at room temperature but not at 0–2 °C. Faggio et al. (1), however, reported an increase in TC after 6 h; an increase in Hb, MCH, and MCHC after 24 h; and a decrease in WBC count after 72 h in EDTA-anticoagulated blood at 4 °C. On the other hand, the results obtained by Faggio et al. (17) showed that storage of heparinized blood at 4 °C for 24 h caused only minor and insignificant changes: an increase in MCH and MCHC (similarly as in our study) and a slight decrease in WBC. Comparison of these results indicates the importance of the anticoagulant used. The results concerning the effects of blood storage on the results of hematological analysis in other vertebrates show that at low temperatures blood is very stable. Cohle et al. (23) reported no changes in Ht, Hb, RBC, MCV, MCH, MCHC, WBC, and TC values of human blood stored at 4 °C for 3 days, while at room temperature a significant increase in Ht and MCV took place after 24 h. Olsen et al. (7) found that Ht and WBC increased in blood of minipigs stored for 25.5 h at 20 °C, while at 5 °C TC values decreased; therefore, the authors concluded that time delay may change the results of analyses and cause increased variation.

The results of the present study showed that heparin concentrations of 10–200 IU/cm^3 may affect the values of hematological parameters in fish; however, the results obtained in two experiments were different. The gradual decrease in erythroblasts, leukocytes, and thrombocytes with the increase in heparin concentration observed in experiment 1 suggests the possible destruction of these cells. However, the results of experiment 2 did not confirm these observations but provided other evidence of the effect of heparin: a decrease in the oxidative metabolic activity of phagocytes and slight alterations in the differential count of thrombocytes. These results and the data obtained by other authors indicate that heparin may alter the results of blood analyses in fish. Therefore, all sampling tubes should be heparinized with equal amounts of anticoagulant, preferably below 50 IU/cm^3. In the case of healthy and unstressed fish it is possible to use only heparinized needles and nonheparinized tubes; therefore, pilot sampling is recommended to test such a possibility. Decreases in RBC, WBC, and TC values in blood stored at room temperature already at 2 h after sampling indicate that blood must be refrigerated immediately after sampling. However, some alterations observed in refrigerated blood within 24 h indicate that most analyses should be performed within less than 1 day after sampling.

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References

1. Faggio C, Casella S, Arfuso F, Marafioti S, Piccione G, Fazio F. Effect of storage time on haematological parameters in mullet, *Mugil cephalus*. Cell Biochem Funct 2013; 31: 412-416.
2. Yonar SM, Ural MS, Silici S, Yonar ME. Malathion-induced changes in the haematological profile, the immune response, and the oxidative/antioxidant status of *Cyprinus carpio carpio*: protective role of propolis. Ecotoxicol Environ Saf 2014; 102: 202-209.
3. Bojarski B, Ludwikowska A, Kurek A, Pawlak K, Tombarkiewicz B, Lutnicka H. Hematological alterations in common carp (*Cyprinus carpio* L.) exposed to herbicides: pendimethalin and ethofumesate tested separately and in mixture. Folia Biol-Krakow 2015; 63: 167-174.
4. Kumar R, Banerjee TK. Arsenic induced hematological and biochemical responses in nutritionally important catfish *Clarias batrachus* (L.). Toxicol Rep 2016; 3: 148-152.

5. Blaxhall PC, Daisley KW. Routine haematological methods for use with fish blood. J Fish Biol 1973; 5: 771-781.

6. Mainwaring G, Rowley AF. The effect of anticoagulants on *Blennius pholis* L. leucocytes. Comp Biochem Physiol A 1985; 80: 85-91.

7. Olsen AK, Bladbjerg EM, Jensen AL, Hansen AK. Effect of pre-analytical handling on haematological variables in minipigs. Lab Anim 2001; 35: 147-152.

8. Korcock DE, Houston AH, Gray JD. Effects of sampling conditions on selected blood variables of rainbow trout, *Salmo gairdneri* Richardson. J Fish Biol 1988; 33: 319-330.

9. Tavares-Dias M, Ragonha Oliveira S. A review of blood coagulation system of fish. Rev Bras Bioci 2009; 7: 205-224.

10. Svobodova Z, Pravda D, Palackova J. Unified Methods of Haematological Examination of Fish. Methods No 20. Vodnany, Czech Republic: Research Institute of Fish Culture and Hydrobiology; 1991.

11. Walencik J, Witeska M. The effects of anticoagulants on hematological indices and blood cell morphology of common carp (*Cyprinus carpio* L.). Comp Biochem Physiol C 2007; 146: 331-335.

12. Casillas E, Smith LS. Effect of stress on blood coagulation and haematology in rainbow trout (*Salmo gairdneri*). J Fish Biol 1977; 10: 481-491.

13. Ruis MAW, Bayne CJ. Effects of acute stress on blood clotting and yeast killing by phagocytes of rainbow trout. J Aquat Anim Health 1997; 9: 190-195.

14. Smit GL, Hattingh J, Schoonbee HB. Observations on some effects of disodium ethylenediamine tetra-acetate and heparin on fish blood. Comp Biochem Physiol C 1977; 57: 35-38.

15. Teixeira de Oliveira A, de Carvalho Santos MQ, Gonzaga Lemos JR, Tavares-Dias M, Marcon JL. Comparison of the effects of anticoagulants used in blood collection to determine blood parameters of free-living stingrays from the *Potamotrygon genus* (Elasmobranchii: Potamotrygonidae) Biota Amazonia 2015; 5: 55-58.

16. Vaz Farias TH, Pereira NL, de Padua SB, de Oliveira Alves L, Sakabe R, de Andrade Belo MA, Pilarski F. Na_2EDTA anticoagulant impaired blood samples from the teleost *Piractus mesopotamicus*. Pesq Vet Bras 2016; 36: 431-435.

17. Faggio C, Arfuso F, Piccione G, Zumbo A, Fazio F. Effect of three different anticoagulants and storage time on haematological parameters of *Mugil cephalus* (Linnaeus, 1758). Turk J Fish Aquat Sci 2014; 14: 1-7.

18. Tavares-Dias M, Silva Sandrim EF. Influence of anticoagulants and blood storage on hematological values in tambaqui, *Colossoma macropomum*. Acta Sci 1998; 20: 151-155.

19. Studnicka M, Siwicki AK, Ryka B. Phagocytic ability of neutrophils in carp (*Cyprinus carpio*). Isr J Aquacult-Bamid 1985; 37: 123-128.

20. Van Vliet KJ, Smit GL, Pieterse JJ, Schoonbee HJ, Van Vuren JHJ. The effects of generally used anticoagulants on the haemolysis of fish erythrocytes. Water SA 1985; 2: 87-92.

21. Nielsen H. Influence of five different anticoagulants on human blood monocyte isolation and functional activities. Acta Path Microbiol Immunol Scand C 1985; 93: 49-52.

22. Sissener Engstad C, Guttenberg TJ, Osterud B. Modulation of blood cell activation by four commonly used anticoagulants. Thromb Haemostasis 1997; 77: 690-696.

23. Cohle SD, Saleem A, Makkaoui DE. Effects of storage of blood on stability of hematologic parameters. Am J Clin Pathol 1981; 76: 67-69.