Does the Site of Blood Collection in Fish Affect Haematological and Blood Biochemical Results?

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Short communication

The values of haematological and selected blood plasma biochemical parameters of juvenile common carp (*Cyprinus carpio* Linnaeus, 1758) were compared between blood samples taken from caudal vein and heart to evaluate the influence of blood sampling body site on the obtained results in two groups of fish of different blood sampling order: I – first by caudal and then by cardiac puncture, II – first by cardiac and then by caudal puncture. The obtained results revealed statistically significant (p<0.05) differences only in group I where red blood cell (RBC) count was higher in caudal vein blood, while haematocrit (Ht) value, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), total protein (TP) concentration, and magnesium (Mg) level were higher in cardiac blood samples. No statistically significant differences occurred in white blood cell (WBC) count, differential leukocyte count or erythrocyte morphology based on stained blood smears. The obtained results showed that blood sampling body site may affect the results of haematological and plasma biochemical analyses.

Key words: Common carp, cardiac puncture, caudal puncture, blood components.

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Haematological examination, often accompanied by determination of plasma or serum biochemical indices, is frequently used to evaluate the effects of environmental factors on fish organisms. The values of haematological parameters in fish may be affected by various natural and anthropogenic factors. They may change with variations in physicochemical parameters of water, such as oxygen concentration (AFFONSO *et al.* 2002), temperature (HE *et al.* 2007), pH (GHANBARI *et al.* 2012) or salinity (FAZIO *et al.* 2013).

The values of haematological indices may be also affected by many other external and internal factors, such as photoperiod, nutritional state, age, gender, and cycle of sexual maturity (FAZIO 2019; AHMED *et al.* 2020). A number of authors reported also haematological alterations in fish due to exposure to environmental contaminants (e.g. DRĄG-KOZAK *et al.* 2020). Haematological examination is often used to evaluate pharmaceutical toxicity (e.g. KONDERA *et al.* 2020) or in assessment of the state of fish organism in the course of parasitic (e.g. WITESKA *et al.* 2010) and infectious diseases (e.g. MCBEATH *et al.* 2015). However, analysis of haematological data in fish is complicated due to the lack of generally accepted reference values for this group of vertebrates. Determination of haematological reference values in fish is much more difficult than in the case of mammals due to poikilothermy and high spatial, temporal and individual variability (WITESKA *et al.* 2016). Moreover, the values of haematological parameters may also vary due to different methods of blood sampling, processing and analysis applied by various scientists (LUGOWSKA *et al.* 2017; BOJARSKI *et al.* 2018). Fish blood is routinely sampled from the caudal vein or from the heart. In the case of small fish, blood can be collected by cutting off the caudal peduncle (SOTOUDEH & JAFARI 2017) or dorsal gill incision (WATSON *et al.* 1989). It was previously demonstrated that the site of blood collection significantly affected basic haematological results (BOJARSKI *et al.* 2018). However, no data are available whether blood sampling from different body sites affects blood biochemical indices. Thus, the aim of this study was to compare haematological parameters (including erythrogram – erythrocyte morphology based on stained blood smears) and selected blood biochemical values of common carp (*Cyprinus carpio* Linnaeus, 1758) in blood sampled by caudal vein puncture and cardiac puncture.

Materials and Methods

The study was approved by the II Local Institutional Animal Care and Use Committee (IACUC) in Kraków, Poland (resolution No. 152/2020). It was performed on 30 sexually immature individuals of clinically healthy common carp (*Cyprinus carpio* L.) of body length 28.0-30.0 cm obtained from the Fisheries Experimental Station of the University of Agriculture in Krakow, Poland in July 2020. The fish were kept for 3 days (acclimation) in plastic flow-through tanks with a constant inflow of water from the Rudawa river (the same river supplies the ponds of the facility from which the fish were obtained). Each 500-liter tank contained 15 individuals. Water quality parameters were as follows: NO_3^- 5-10 mg/l, NO_2^- 0.05-0.1 mg/l, NH_3 0 mg/l, O_2 4-5 mg/l, pH 7.5-7.8, and temperature 19.1-19.4°C. Except for the temperature, the values of parameters were measured with aquarium kits produced by Zoolek (Poland) or Sera (Germany) companies. The fish were fed daily (2% of their body weight) with commercial dry pellets. After the acclimation, the fish were randomly divided into two equinumerous (n=15) groups. The blood was sampled without the use of anaesthetics. From the fish of group I the blood was collected first from the caudal vein and next from the heart, while in the case of group II, first the blood was sampled from the heart and then from the caudal vein. Blood samples from the fish of both groups were taken simultaneously and the procedure was carried out by staff experienced in collecting blood samples from common carp. Blood sampling from each individual took approximately 2 mins (in total). The blood was taken with previously heparinized plastic syringes of 1 ml volume into heparinized Eppendorf tubes. During the procedure of sampling, the biological material was kept refrigerated. Part of the collected blood was centrifuged (3000 g, 20 min) to obtain plasma, and then stored at -20°C until biochemical analyses were performed. Haematological examinations were performed using the whole blood in 24 hours after sampling. The blood samples were stored at +4°C until the haematological analyses were performed.

Haematological analyses

The blood was gently mixed using tube roller mixer before the analyses. The following haematological parameters were evaluated: red blood cell (RBC) count, haematocrit (Ht) value, haemoglobin (Hb) concentration, and white blood cell (WBC) count. Derived red blood parameters: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were calculated using Ht, RBC, and Hb values, according to the formulas: MCV = (Ht × 10) / RBC, MCH = Hb / RBC, and MCHC = (Hb × 100) / Ht. Blood smears were also made and stained with May-Grünwald and Giemsa solutions for evaluation of differential leukocyte count and erythrocyte morphology. Ht was measured using microhaematocrit method: capillaries with blood with one end sealed were centrifuged for 5 minutes at 12,879 g and the values were measured using haematocrit reader. RBC and WBC values were obtained using Bürker haemocytometer. Blood was diluted 1:100 with Hayem's solution and introduced into the haemocytometer chamber. Erythrocytes and leukocytes were counted in the appropriate squares under ×400 magnification and cell count values were calculated. Hb content was measured using spectrophotometric cyanmethaemoglobin method. Blood was diluted with Drabkin's solution, extinction of cyanmethaemoglobin was read at the 540 nm wavelength and Hb was calculated using the equation of relationship between the extinction

and concentrations of standard haemoglobin solutions. Stained blood smears were viewed under ×400 magnification: 300 erythrocytes and 100 leukocytes were inspected to evaluate erythrocyte morphology and to obtain leukocyte differential count (percentage of various cell types). Each analysis was done by the same person to avoid bias.

Biochemical analyses

Blood plasma was used to measure concentrations of glucose (Glu), total protein (TP), cholesterol (Chol), calcium (Ca), and magnesium (Mg). These indices were determined using BioSystems assays (BioSystems S.A., Barcelona, Spain) with EPOCH (BioTek, USA) spectrophotometer. The amount of glucose was measured using glucose oxidase with an alternative oxygen acceptor and peroxidase. Absorption intensity of coloured product (quinoneimine) was determined at 500 nm wavelength. Total protein concentration was determined using the biuret reaction with copper (II) ion in alkaline medium forming a coloured complex that can be measured spectrophotometrically at 545 nm. Free and esterified cholesterol concentrations were determined by means of the coupled enzymatic reactions using cholesterol esterase, cholesterol oxidase and peroxidase. The colour intensity of quinoneimine (a final product) is proportional to the concentration of cholesterol and can be determined at 500 nm. Determination of calcium concentration was performed using reaction with methylthymol blue in alkaline medium. Absorption intensity of coloured complex was measured at 610 nm wavelength. Likewise, magnesium reacts with xylidyl blue in alkaline medium forming a coloured complex, that can be determined at 520 nm. All biochemical measurements were carried out in triplicates in order to minimize the measurement error.

Statistical analyses

The compliance of the obtained results with the normal distribution was tested by the Shapiro-Wilk test. In the case of dependent samples under the assumption that they were normally distributed, the paired two-sample t-test was applied, while without that assumption the Friedman test was applied. In the case of independent samples under the assumption that they were normally distributed the unpaired two-sample t-test was applied, while without that assumption the two-sample Wilcoxon test was applied. The significance level was set at 0.05. The statistical calculations were performed using free software R. Two groups, i.e. group I (caudal vein-heart) and group II (heart-caudal vein), each containing 15 fish were used for evaluating every parameter studied except for evaluating RBC count, Ht value, Hb concentration, MCV, MCH, MCHC, and WBC count, where group I contained 12 fish and group II contained 5 fish due to blood clots. Data are presented as means ± SD.

Results

In both groups of fish blood clots were observed in the blood samples. In group I no clots occurred in caudal vein samples and 3 samples clotted after cardiac puncture, while 2 cardiac samples and 9 caudal samples clotted in group II. Clotted samples were excluded from RBC, Ht, Hb, and WBC measurements and MCV, MCH, and MCHC calculations. Therefore, these parameters were compared for 12 individuals in group I and 5 individuals in group II.

The results of haematological analyses revealed some statistically significant differences in group I (Table 1). RBC was higher in caudal vein samples, while Ht, MCV, and MCH were higher in cardiac samples. No significant differences were observed in group II. WBC in group I tended to be higher in caudal samples but the difference was insignificant due to individual variability. Similarly, no significant differences occurred in differential leukocyte count or erythrocyte morphology (Table 2). Abnormal erythrocytes detected in the samples included mainly the cells undergoing haemolysis (from 5.20±6.61 in caudal samples in group I to 12.60±10.23 in caudal samples in group II). The cells showing abnormal shape, deformed nucleus, cytoplasm vacuolation were very scarce (Table 2). The values of biochemical parameters were significantly different only in group I where higher concentrations of TP and Mg occurred in cardiac samples compared to the caudal vein blood (Table 3).

Discussion

Blood sampling is necessary to perform haematological tests in the assessment of fish health (CLAUSS et al. 2008) as well as for many scientific studies (e.g. BURGOS-ACEVES et al. 2019). According to GROFF & ZINKL (1999), in the case of fish it is possible to collect safely about 30-50% of the blood volume at one time. Fish blood is routinely collected from the caudal vein (KREUTZ et al. 2011; VELISEK et al. 2013; BOAVENTURA et al. 2020; LI et al. 2021) or from the heart (PRIYA et al. 2015; WITESKA et al. 2016; KONDERA et al. 2020). According to BOJARSKI et al. (2018), blood sampling from the heart is an easier and faster method which allows to obtain more blood in a shorter time. On the other hand, in the present study a higher number of venous blood clots was detected in the case of fish that had been previously subjected to cardiac puncture. In the case of fish whose blood was taken in the opposite order, i.e. first from their caudal vein and next from their heart the number of clots was much lower. This indicates that the increased number of clots in caudal samples was a result of stress caused by cardiac puncture.

Table 1

Haematological parameters of common carp (mean±SD). Group I: blood collected first from the caudal vein and next from the heart (n=12); Group II: blood collected first from the heart and next from the caudal vein (n=5). Parameters within the same group compared with the paired two-sample t-test; parameters from different groups compared with the unpaired two-sample t-test. A, B, C, D – means with no letter in common are significantly different (p<0.05)

Group Parameter	Group I		Group II	
	Caudal vein	Heart	Heart	Caudal vein
RBC (10^6/μl)	1.33±0.27[B]	1.14±0.20[A]	1.08±0.15[A]	1.26±0.32[AB]
Ht (%)	29.04±5.41[A]	31.50±6.25[B]	29.80±4.67[AB]	30.20±4.86[AB]
Hb (g/l)	79.66±17.57[A]	80.55±13.87[A]	68.93±12.00[A]	78.77±23.21[A]
MCV (fl)	221.35±28.93[A]	276.82±33.00[B]	278.05±38.60[B]	245.34±35.04[AB]
MCH (pg)	60.61±9.72[A]	71.45±10.78[B]	64.11±7.97[AB]	63.15±11.58[AB]
MCHC (g/l)	274.25±33.03[B]	258.92±29.06[AB]	231.83±24.36[A]	257.85±37.39[AB]
WBC (10^3/μl)	37.33±15.39[A]	33.45±14.36[A]	27.64±8.80[A]	29.86±9.87[A]

RBC – red blood cell count; Ht – haematocrit value; Hb – haemoglobin concentration; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; WBC – white blood cell count.

Table 2

Differential leukocyte count and erythrocyte morphology of common carp (mean±SD). Group I: blood collected first from the caudal vein and next from the heart (n=15); Group II: blood collected first from the heart and next from the caudal vein (n=15). Parameters within the same group compared with the Friedman test; parameters from different groups compared with the two-sample Wilcoxon test. A, B, C, D – means with no letter in common are significantly different (p<0.05)

Group Parameter	Group I		Group II	
	Caudal vein	Heart	Heart	Caudal vein
Lym	87.67±5.81[CD]	89.53±2.88[BC]	80.33±11.52[AD]	78.33±12.28[AB]
Myelo	9.73±4.67[AD]	8.53±2.45[AB]	17.20±12.02[CD]	19.00±11.96[BC]
Meta	1.13±1.36[A]	0.73±0.59[A]	0.93±1.03[A]	1.40±1.80[A]
Band	0.07±0.26[A]	0.07±0.26[A]	0.13±0.35[A]	0.07±0.26[A]
Segm	0.60±0.91[A]	0.47±0.83[A]	0.60±0.91[A]	0.60±1.06[A]
Eos	0.07±0.26[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]
Mono	0.73±1.28[A]	0.67±1.05[A]	0.80±0.94[A]	0.60±0.83[A]
CSE	0.73±0.80[A]	0.80±0.56[A]	1.80±2.21[A]	1.00±1.25[A]
CSEN	0.13±0.35[A]	0.07±0.26[A]	0.13±0.35[A]	0.00±0.00[A]
ECV	1.00±1.25[A]	1.33±1.84[A]	0.47±0.64[A]	0.47±0.74[A]
EH	5.20±6.61[A]	5.67±7.43[AB]	7.20±7.75[AB]	12.60±10.23[B]
UE	292.93±6.75[B]	292.13±7.15[AB]	290.40±7.67[AB]	285.93±9.47[A]

Lym – lymphocytes, Myelo – myelocytes; Meta – metamyelocytes; Band – band neutrophils; Segm – segmented neutrophils; Eos – eosinophils; Mono – monocytes; CSE – changed shape of erythrocytes; CSEN – changed shape of erythrocyte nucleus; ECV – erythrocyte cytoplasm vacuolization; EH – erythrocyte haemolysis; UE – unchanged erythrocytes.

Table 3

Blood biochemical parameters of common carp (mean±SD). Group I: blood collected first from the caudal vein and next from the heart (n=15); Group II: blood collected first from the heart and next from the caudal vein (n=15). Parameters within the same group compared with the paired two-sample t-test; parameters from different groups compared with the unpaired two-sample t-test. A, B, C, D – means with no letter in common are significantly different (p<0.05)

Group Parameter	Group I		Group II	
	Caudal vein	Heart	Heart	Caudal vein
Glu (mg/dl)	128.79±19.61[B]	133.37±33.40[B]	130.55±39.75[AB]	112.20±20.44[A]
TP (g/dl)	19.31±3.66[A]	23.25±5.09[B]	24.31±7.07[B]	24.38±4.13[B]
Chol (mg/dl)	219.45±56.10[A]	226.39±62.17[A]	186.86±47.05[A]	195.00±34.00[A]
Ca (mg/dl)	5.53±1.12[B]	5.31±1.60[AB]	4.57±1.11[A]	4.48±1.06[A]
Mg (mg/dl)	2.51±0.43[A]	3.38±0.70[B]	3.19±0.78[B]	3.45±0.87[B]

Glu – glucose; TP – total protein; Chol – cholesterol; Ca – calcium; Mg – magnesium.

In the present study significantly higher RBC number was noted in venous blood samples in comparison to cardiac ones, while the values of Ht, MCV, and MCHC were higher in the case of blood collected from the heart in fish bled first from the caudal vein and then from the heart. The study comparing haematological values between venous and cardiac blood of *Cyprinus carpio* juveniles of body mass 80±10 g and length 15-16 cm previously performed by BOJARSKI *et al.* (2018) showed that RBC count, Ht value, and Hb concentration were significantly higher in venous samples compared to cardiac ones, while derived red blood cell indices (MCV, MCH, and MCHC) did not significantly differ. Percentages of abnormal erythrocytes, as well as white blood cell parameters did not significantly differ between the caudal and cardiac samples in both groups. It was stated that the observed differences may have resulted from the fact that blood in the caudal vein was more concentrated due to fluid loss in the capillary system, while cardiac blood was diluted due to fluid return from the secondary vascular system (SVS) or the SVS might have sequestrated red blood cells from the blood circulation (BOJARSKI *et al.* 2018). The differences in Ht, MCV, and MCHC values recorded in the current research are difficult to explain. The discrepancy between the present results and those obtained in the study previously mentioned may be a result of different size/weight and age of fish.

Literature data indicates that the method of blood collection in fish can affect the blood biochemical results obtained. In the present study, higher concentrations of total protein and magnesium were found in the plasma of blood collected from the heart compared to samples taken from the caudal vein. As in the case of haematological results, it was observed only in fish, whose blood was collected from the caudal vein at first and later from the heart. In order to explain biological mechanisms of the differences observed further studies are necessary. The fact that the results of blood biochemical analyses depend on the site of blood collection was confirmed by the study performed by WATSON *et al.* (1989), who revealed that plasma aspartate aminotransferase (AST) and creatine kinase (CK) activities in caudal peduncle transection samples of bluegill (*Lepomis macrochirus*) blood were significantly higher in comparison to the levels obtained by dorsal gill incision. The blood biochemistry results obtained in the current study were different from those previously reported in koi carp (*Cyprinus carpio*). The values of glucose and total protein concentrations recorded in the present study were visibly (2-3 times or 6-8 times, respectively) higher than the values obtained by TRIPATHI *et al.* (2003) in koi carp, while cholesterol and magnesium concentrations were similar. The concentration of calcium recorded in the current study was about two times lower than the values revealed by TRIPATHI *et al.*

(2003). However, the reference haematological values for common carp (*Cyprinus carpio*) obtained by WITESKA *et al.* (2016) (RBC 1.35-1.51 $\times$ 10^6/µl, Ht 24.0-25.5%, Hb 62.4-69.6 g/l, MCV 178.6-200.1 fl, MCH 47.3-53.1 pg, MCHC 256.6-284.9 g/l, WBC 51.3-60.8 $\times 10^3$/µl, frequency of lymphocytes 87.3-91.7%, frequency of neutrophils 5.6-8.9%, and frequency of monocytes 0.7-2.0%) were in most cases similar with those observed in the current study.

The results presented in the current paper confirm previous studies as they show that the site of blood collection has an influence on the obtained haematological results. This is of particular importance for red blood cell parameters as well as for some blood biochemical indices, which varied significantly depending on whether blood was taken from the heart or from the caudal vein. Due to these facts, it is not recommended to compare the results obtained on the basis of analyses of blood collected from different sites, not even in the case of fish of the same species.

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Author Contributions

Research concept and design: B.B., M.S., M.W.; Collection and/or assembly of data: B.B., M.S., E.D.-K., A.R.-B., S.K., L.S., E.K., K.Ł., M.W.; Data analysis and interpretation: B.B., L.S., M.W.; Writing the article: B.B., A.R.-B., L.S., M.W.; Critical revision of the article: B.B., M.S., E.D.-K., A.R.-B., S.K., L.S., E.K., K.Ł., M.W.; Final approval of article: B.B., M.S., E.D.-K., A.R.-B., S.K., L.S., E.K., K.Ł., M.W.

Conflict of Interest

The authors declare no conflict of interest.

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