FISHERIES & AQUATIC LIFE (2023) 31: 24 - 30
Archives of Polish Fisheries
DOI 10.2478/aopf-2023-0003

sciendo

RESEARCH ARTICLE

Does the site of blood collection and sex of crucian carp (*Carassius carassius*) affect hematological and blood biochemical results?

Bartosz Bojarski, Magdalena Socha, Leszek Szała, Denisa Medkova, Aneta Hollerova, Ewa Drąg-Kozak, Sylwia Zając, Agnieszka Rombel-Bryzek

Received – 27 July 2022/Accepted – 12 January 2023. Published online: 31 March 2023;
Citation: Bojarski, B., Socha, M., Szała, L., Medkova, D., Hollerova A., Drąg-Kozak, E., Kapinos, S., Rombel-Bryzek, A. (2023). Does the site of blood collection and sex of crucian carp (*Carassius carassius*) affect hematological and blood biochemical results? Fisheries & Aquatic Life 31, 24-30

Abstract. Blood analysis is used to evaluate the effects of various environmental factors on fishes and to assess fish health and welfare. Fish blood is routinely sampled from the caudal vein or the heart. The aim of the present study was to determine the differences between venous and cardiac crucian carp (*Carassius carassius* (L.)) blood parameters. Additionally, the influence of sex was tested. The fish were divided into two groups: group I consisted of 10 males and 9 females, group II consisted of 8 males and 8 females. The fish from group I had blood sampled first from the caudal vein and then from the heart. The fish from group II had the procedures in the opposite order. The hematological parameters determined in the present study depended on the blood collection site and the sex of the individuals. Blood biochemical indices depended on the sex of the fish but not on the blood sampling site. The effect of sampling order was observed in the case of both hematological

B. Bojarski [✉]
Institute of Ichthyobiology and Aquaculture in Gołysz, Polish Academy of Sciences, Kalinowa 2, Zaborze, 43-520 Chybie, Poland
E-mail: bbojarski@o2.pl, bartosz.bojarski@golysz.pan.pl

M. Socha
Department of Animal Physiology and Endocrinology, Faculty of Animal Sciences, University of Agriculture in Kraków, Mickiewicza 24/28, 30-059 Kraków, Poland

L. Szała
Department of Mathematics, Informatics and Cybernetics, Faculty of Chemical Engineering, University of Chemistry and Technology, Prague, Technická 5, 166 28 Praha 6, Czech Republic

D. Medkova
Department of Animal Protection and Welfare & Veterinary Public Health, Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic
Department of Animal Breeding, Animal Nutrition and Biochemistry, Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic
Department of Zoology, Fisheries, Hydrobiology and Apiculture, Faculty of Agrisciences, Mendel University in Brno, Zemědělská 1665/1, 613 00 Brno, Czech Republic

A. Hollerova
Department of Animal Protection and Welfare & Veterinary Public Health, Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic
Department of Infectious Diseases & Preventive Medicine, Veterinary Research Institute, Hudcova 296/70, 621 00 Brno, Czech Republic

E. Drąg-Kozak
Department of Animal Nutrition and Biotechnology, and Fisheries, Faculty of Animal Sciences, University of Agriculture in Kraków, Mickiewicza 24/28, 30-059 Kraków, Poland

S. Zając, A. Rombel-Bryzek
Department of Clinical Biochemistry and Laboratory Diagnostics, Institute of Medical Sciences, University of Opole, Oleska 48, 45-052 Opole, Poland

and biochemical parameters. The results of the current study indicated that the blood collection site, the sex of fish, and sampling order should be taken into consideration in hematological experiments.

Keywords: crucian carp, blood sampling, blood parameters, methodology

Introduction

Hematological analysis, often accompanied by determinations of plasma or serum biochemical parameters, is frequently used to evaluate the effects of various environmental factors (e.g., toxic agents) on fish during scientific studies and to assess fish health and welfare in aquaculture (Fazio 2019, Ahmed et al. 2020, Bojarski and Witeska 2020, Bojarski et al. 2020, Witeska et al. 2022). It is worth mentioning that the values of hematological and blood biochemical parameters in fish depend on various endogenous factors, among others age (Hrubec et al. 2001, Ejraei et al. 2015, Okoye et al. 2016, Melillo-Filho et al. 2021) and sex (Motlagh et al. 2012, Charoo et al. 2013, Zakęś et al. 2016, Suljević and Mitrasinović-Brulić 2020). Moreover, it was demonstrated that blood indices can vary with different blood sampling methods (Bojarski et al. 2018, 2021) and analyses (Lugowska et al. 2017). Fish blood is routinely sampled from the caudal vein or from the heart (Bojarski et al. 2018, 2021). To date, very few scientific papers have been published on the differences between blood parameters depending on the sampling site. Bojarski et al. (2018) compared hematological parameters between venous and cardiac blood of common carp (*Cyprinus carpio* L.) and showed that erythrocyte count, hematocrit value, and hemoglobin concentration were higher in venous samples compared to cardiac samples, while other indices (mean cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, erythroblast frequency, abnormal erythrocyte frequency, leukocyte count, percentage of lymphocytes, percentage of neutrophils, percentage of monocytes, and thrombocyte count) did not differ significantly. A similar study performed by Bojarski et al. (2021) demonstrated that red blood cell count was higher in the caudal vein blood of common carp (*C. carpio*), while hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, and total protein and magnesium concentrations were higher in cardiac blood samples. To our knowledge, there is no data on the differences between venous and cardiac blood in the context of other species of cyprinids. Thus, the main goal of the current study was to determine the differences between venous and cardiac crucian carp, *Carassius carassius* (L.) hematological and blood biochemical parameters. Additionally, the factor of sex was taken into consideration.

Material and Methods

Experimental design

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 35 sexually mature individuals of clinically healthy crucian carp (*Carassius carassius*). The fish weighed 270.23 ± 65.77 g (mean ± SD) and had a length of 22.50 ± 1.48 cm (mean ± SD). Before the blood sampling, they were kept for 7 days (acclimation) in plastic flow-through tanks with a constant inflow of water from the Rudawa River. Each 500-liter tank contained 8–9 individuals. Water quality parameters were as follows: NO_3^- 10 mg l^{-1}, NO_2^- 0.05 mg l^{-1}, NH_3 0.04 mg l^{-1}, PO_4^{3-} 0.25 mg l^{-1}, pH 8.0, general hardness 20°GH, carbonate hardness 14°KH, and temperature 8.0°C. Except for temperature (which was measured with a thermometer), the values of the tested parameters were determined with Zoolek (Poland) colorimetric kits. The fish were not fed because of the low water temperature (the experiment was conducted in November 2021). After acclimation, the fish were divided randomly into two groups: group I consisted of 10 males and 9 females, group II consisted of 8 males and 8 females. The fish from group I had blood sampled first from the caudal

vein and then from the heart. The fish from group II had the procedures in the opposite order.

Blood sampling

Blood was sampled by staff experienced in collecting blood samples from freshwater fish. Blood sampling from each individual took approximately 2–3 minutes and was done without an anesthetic. The blood was drawn with previously heparinized plastic syringes of 1 ml volume into heparinized Eppendorf tubes. During sampling, the biological material was kept refrigerated (4°C). Part of the collected blood was centrifuged (3000 g, 5 min) to obtain plasma, and then stored at -80°C until biochemical analyses were performed.

Hematological analysis

The following hematological indices were evaluated: red blood cell (RBC) count, hematocrit (Hct) value, hemoglobin (Hb) concentration, and white blood cell (WBC) count. Next, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using standard formulas (Bomski 1983). RBC and WBC counts were determined with a Bürker hemocytometer and a standard light microscope and 200x magnification for erythrocyte analysis and 400x for that of leukocytes. Before counting, the blood was diluted 100 times with Hayem's solution. Hct was measured using the microhematocrit method – capillaries with blood were centrifuged for 5 minutes at 15,717 g and the values were measured using a standard hematocrit reader. Hb content was determined using the cyanmethemoglobin method. Absorbance was read at 540 nm. Each analysis was done by the same person.

Biochemical analysis

The concentration of total protein (TP), glucose (Glu), cholesterol (Chol), calcium (Ca), and magnesium (Mg) in the blood plasma of the fish was determined spectrophotometrically with BioSystems assays (BioSystems S.A., Barcelona, Spain) as previously described (Bojarski et al. 2021).

Statistical analysis

The compliance of the data analyzed with normal distribution was verified using the Shapiro-Wilk test. The homogeneity of variances was verified using Levene's test. The significance level was 0.05 for both tests. In most cases the tests did not contradict the necessary assumptions. The homogeneity of covariances was verified using Box's M-test. Since Box's M-test is highly sensitive, the significance level was 0.001 (the homogeneity of covariances was violated in only one case). Every parameter studied was analyzed using two-way mixed ANOVA. The between subject factor was sex (with two possible values – male or female) and the within subject factor was blood collection site (with two possible values – caudal vein or heart). The significance level for each test was 0.05. If the two-way interaction was significant, then the one-way model of the between subject factor (sex) at each level of the within subject factor (blood collection site) was applied. The result of the test was interpreted using the Bonferroni correction, i.e., p.adj = 2 * p. If the two-way interaction was not significant, the simple main effects of each of the two variables of sex and blood collection site were analyzed. The main effect of sex was analyzed using the independent sample t-test and the main effect of the blood collection site was analyzed using the dependent sample t-test. The level of significance of each t-test was 0.05.

Results

Group I

The blood collection site significantly influenced several hematological parameters. Nevertheless, the effect of this factor on biochemical indices was not confirmed (Table 1). Post hoc analysis showed that

Table 1
Hematological and blood biochemical parameters of crucian carp (*Carassius carassius*) from which blood was collected first from the caudal vein and next from the heart (group I) (mean ± SD)

Parameter	Unit	Caudal vein		Heart		ANOVA, p-value		
		Male	Female	Male	Female	BCS	sex	interactions
RBC	10^6 µl^{-1}	1.19 ± 0.142	1.15 ± 0.098	1.13 ± 0.119	0.98 ± 0.193	0.020	0.065	0.270
Hct	%	36.40 ± 3.071	31.39 ± 1.816	29.15 ± 5.432	26.83 ± 5.540	0.000	0.025	0.308
Hb	g dl^{-1}	13.82 ± 1.489	13.20 ± 2.585	11.91 ± 1.764	11.04 ± 2.705	0.029	0.166	0.886
MCV	fl	310.97 ± 54.232	275.68 ± 29.094	260.85 ± 52.065	279.41 ± 64.402	0.156	0.646	0.103
MCH	pg	118.58 ± 25.342	115.25 ± 20.566	106.64 ± 18.151	113.40 ± 23.891	0.334	0.821	0.476
MCHC	g dl^{-1}	38.13 ± 4.285	42.47 ± 10.533	41.61 ± 5.952	41.14 ± 5.024	0.680	0.292	0.363
WBC	10^3 µl^{-1}	17.75 ± 5.490	22.92 ± 4.050	16.38 ± 5.152	18.19 ± 3.909	0.056	0.042	0.276
TP	g l^{-1}	34.61 ± 6.134	33.14 ± 4.607	34.10 ± 4.668	32.68 ± 5.259	0.735	0.463	0.987
Glu	mg dl^{-1}	145.99 ± 100.981	140.03 ± 59.076	163.97 ± 112.467	142.61 ± 58.775	0.237	0.733	0.372
Chol	mg dl^{-1}	458.22 ± 141.130	475.97 ± 133.523	468.18 ± 174.571	433.28 ± 160.174	0.606	0.894	0.410
Ca	mg dl^{-1}	6.08 ± 0.635	8.17 ± 0.937	6.21 ± 0.975	7.86 ± 1.006	0.571	0.000	0.156
Mg	mg dl^{-1}	3.87 ± 0.430	3.43 ± 0.249	3.77 ± 0.328	3.31 ± 0.206	0.182	0.002	0.851

BCS – blood collection site, RBC – red blood cell count, Hct – hematocrit value, Hb – hemoglobin concentration, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, WBC – white blood cell count, TP – total protein, Glu – glucose, Chol – cholesterol, Ca – calcium, Mg – magnesium

venous blood had a significantly higher RBC count, Hct value, and Hb concentration in comparison to cardiac samples (P<0.05). The sex factor had a statistically significant effect on the Hct level, the WBC count, and Ca and Mg concentrations (Table 1). Post hoc analysis showed that the Hct level was significantly higher in male blood samples, while the WBC count was higher in female blood samples (P<0.05). Moreover, the Ca concentration was significantly higher in females, while the Mg level was higher in males (P<0.05).

Group II

As in the case of group I, some hematological parameters of the fish from group II were significantly influenced by the blood collection site, but no impact of this factor on biochemical indices was demonstrated (Table 2). Post hoc analysis showed that blood collected from the heart had statistically significantly more erythrocytes, while venous blood had significantly higher MCV and MCH values (P<0.05). The factor of sex had a statistically significant effect on one hematological parameter (MCH) and two biochemical indices – cholesterol and calcium concentrations (Table 2). Post hoc analysis showed that MCH and cholesterol level were significantly higher in male blood, while Ca concentration was higher in that of females (P<0.05). Moreover, an interaction of both factors studied (blood collection site and sex) was also found for hemoglobin (Table 2), however, post hoc analysis showed no differences between the groups (P<0.05).

Discussion

Hematological parameters tested in the present study (except for MCHC) depended on the blood collection site or/and the sex of the individuals. The venous blood samples had higher Hct values, Hb concentrations, and MCV and MCH values than did those taken from the heart. These results corresponded only partially with the results Bojarski et al.

Table 2
Hematological and blood biochemical parameters of crucian carp (*Carassius carassius*) from which blood was collected first from the heart and then from the caudal vein (group II) (mean ± SD)

Parameter	Unit	Caudal vein		Heart		ANOVA, p-value		
		Male	Female	Male	Female	BCS	sex	interactions
RBC	10^6 µl^{-1}	1.02 ± 0.162	1.02 ± 0.206	1.07 ± 0.316	1.32 ± 0.306	0.023	0.249	0.086
Hct	%	32.19 ± 6.611	32.56 ± 7.093	29.13 ± 6.479	30.63 ± 5.598	0.173	0.736	0.751
Hb	g dl^{-1}	14.01 ±2.240	11.20 ± 2.964	12.49 ±3.244	12.22 ± 2.833	0.517	0.280	0.005
MCV	fl	314.64 ± 31.713	328.19 ± 86.058	279.81 ± 36.768	241.61 ± 62.684	0.037	0.355	0.341
MCH	pg	138.02 ± 12.022	112.56 ± 32.985	119.29 ± 17.413	96.09 ± 26.562	0.022	0.025	0.871
MCHC	g dl^{-1}	44.05 ± 3.511	36.60 ± 14.527	42.65 ± 3.192	39.58 ± 2.965	0.768	0.090	0.417
WBC	10^3 µl^{-1}	18.91 ± 8.357	15.00 ± 2.673	17.81 ± 7.698	16.25 ± 4.381	0.949	0.358	0.346
TP	g l^{-1}	32.18 ± 7.348	28.04 ± 6.303	34.32 ± 8.160	28.88 ± 7.434	0.470	0.142	0.750
Glu	mg dl^{-1}	177.94 ± 74.249	150.57 ± 72.288	171.21 ± 81.024	168.29 ± 82.137	0.738	0.674	0.460
Chol	mg dl^{-1}	512.76 ± 136.025	344.12 ± 90.461	456.41 ± 164.719	366.35 ± 95.348	0.563	0.036	0.194
Ca	mg dl^{-1}	6.14 ± 0.774	8.00 ± 0.884	5.65 ± 0.699	8.07 ± 1.171	0.226	0.000	0.112
Mg	mg dl^{-1}	3.83 ± 0.970	3.49 ± 0.274	3.71 ± 0.765	3.70 ± 0.416	0.631	0.598	0.085

BCS – blood collection site, RBC – red blood cell count, Hct – hematocrit value, Hb – hemoglobin concentration, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, WBC – white blood cell count, TP – total protein, Glu – glucose, Chol – cholesterol, Ca – calcium, Mg – magnesium

(2018) obtained, which could be a consequence of the physiological differences between crucian carp (*C. carassius*) and common carp (*C. carpio*). In contrast to the current experiment, Mikulikova et al. (2022) observed no differences in hematological indices between the blood of rainbow trout (*Oncorhynchus mykiss* (Walbaum)) obtained via caudal and heart punctures. The present study showed that the sex of the crucian carp also affected hematological parameters. Blood collected from males had higher Hct and MCH values, while that of females had more leukocytes. Moreover, the different results obtained between fish from groups I or II suggested that the sampling order had a significant influence on the hematological parameters examined. This indicates it is necessary to consider this factor in hematological studies aimed at comparing the parameters of blood taken from different sites.

The biochemical indices tested in the present experiment did not depend on the blood collection site. On the other hand, Watson et al. (1989) obtained blood samples from bluegill sunfish (*Lepomis macrochirus* (Raf.)) by caudal peduncle transection and dorsal gill incision and demonstrated that plasma aspartate aminotransferase (AST) and creatine kinase (CK) levels in samples obtained by caudal peduncle transection were significantly elevated above the levels obtained in samples collected by dorsal gill incision. Some of the blood biochemical parameters tested in the current study depended on the sex of the fish. Cholesterol and magnesium concentrations were higher in the blood plasma of males, while the calcium concentration was higher in females. The sampling order affected the biochemical results in males, but not in females.

To the best of our knowledge, studies on the influence of blood collection site on the results of hematological and biochemical analyses are rarely conducted, but the effect of sex has been better studied. Motlagh et al. (2012) found significant sex-dependent differences for some hematological parameters in Siamese fighting fish, *Betta splendens* Regan. The authors demonstrated that WBC counts and lymphocyte percentages in females were significantly higher than in males, while the percentage of heterophils was significantly higher in males. Suljević and Mitrasinović-Brulić (2020) observed significantly higher total leukocyte counts and

segmented neutrophil levels in the blood of male brook trout, *Salvelinus fontinalis* (Mitchill)) compared to that of females. Zakęś et al. (2016) compared blood parameters between male and female individuals of pikeperch, *Sander lucioperca* (L.) and observed significantly higher Ht values, Hb concentrations, WBC counts, and MCH and MCHC values in males compared to females. Some scientific articles have reported that not only are hematological indices associated with the sex of fishes but so are the biochemical parameters of blood. Charoo et al. (2013) revealed that total protein, albumin, glucose, phosphorous, and magnesium concentrations in the serum of rainbow trout (*O. mykiss*) were significantly higher in males than in females. Moreover, Zakęś et al. (2016) demonstrated that glucose levels in the blood plasma of pikeperch (*S. lucioperca*) were significantly higher in males than in females, but cholesterol concentration was significantly higher in females. On the other hand, Ejraei et al. (2015) revealed that there were no significant differences in hematological parameters or plasma biochemical indices between male and female grass carp, *Ctenopharyngodon idella* (Val.).

To the best of our knowledge, the current results are the first contribution regarding the issue of the influence of the tested factors on crucian carp blood indices. For this reason, they should be considered preliminary. The mechanisms responsible for the differences observed in the present study are currently unknown, thus, further research is needed to explain the physiological basis of the phenomena observed.

Conclusion

The results of the present study indicated that the blood collection site and sex might affect hematological parameters. Biochemical indices determined in the blood plasma depended on sex but not on the blood collection site. Sampling order was observed to affect both hematological and biochemical parameters. These results and those of the studies cited above indicated that it is necessary to consider these factors in scientific studies. The differences observed in the present experiment could be the subject of further study to explain the physiological mechanisms of the phenomena observed.

Acknowledgments. The study was supported by PROFISH (Grant No. CZ.02.1.01/0.0/0.0/16_019/0000869), the University of Veterinary Sciences Brno, Mendel University in Brno, the Veterinary Research Institute in Brno, the Institute of Ichthyobiology and Aquaculture in Gołysz, and the University of Agriculture in Kraków (subvention no. SUB-020002-D015 and SUB-215-D202).

Author contributions. B.B. idea of the study; B.B., M.S., E. D.-K. experiment design; B.B., M.S., D.M., A.H., E. D.-K., S.Z., A. R.-B. laboratory analyses; L.S. statistical analysis; B.B., M.S., L.S., E. D.-K. data analysis and interpretation; B.B., L.S. writing the manuscript; B.B., M.S., D.M., A.H., E. D.-K., S.Z., A. R.-B. manuscript revising; M.S. cooperation with the Local Ethics Committee; L.S. initial language correction; B.B., M.S., L.S., D.M., A.H., E. D.-K., S.Z., A. R.-B. final approval of the manuscript; M.S. supervising the study; B.B. corresponding author.

ORCID iD

Bartosz Bojarski: https://orcid.org/0000-0003-3922-0329
Magdalena Socha: https://orcid.org/0000-0003-2466-8542
Leszek Szała: https://orcid.org/0000-0003-0772-3124
Denisa Medkova: https://orcid.org/0000-0002-3690-3071
Aneta Hollerova: https://orcid.org/0000-0002-5790-9923
Ewa Drąg-Kozak: https://orcid.org/0000-0002-2572-0034
Sylwia Zając: https://orcid.org/0000-0001-9980-1403
Agnieszka Rombel-Bryzek: https://orcid.org/0000-0001-8790-3541

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