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Seasonal Dynamics of Hematological Parameters in Three Freshwater Fish Species from Aleksandar Stamboliyski Reservoir (Bulgaria)

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Abstract

Seasonal variability can complicate the interpretation of fish hematological parameters used in environmental assessment. We examined seasonal changes in erythrocyte count (RBC), hemoglobin concentration (Hb), hematocrit (PCV), and erythrocyte morphology in common carp (*Cyprinus carpio* Linnaeus, 1758), Prussian carp (*Carassius gibelio* (Bloch, 1782)), and European perch (*Perca fluviatilis* Linnaeus, 1758) from the Aleksandar Stamboliyski Reservoir, Bulgaria. Ten individuals of each species were sampled per season. Water temperature, pH, electrical conductivity, dissolved oxygen, nutrients, and selected metals were also measured. Season significantly affected most hematological variables, although the patterns differed among species. RBC remained comparatively stable in common carp, decreased during summer and autumn in Prussian carp, and was the lowest during autumn in European perch. Hb and PCV reached their highest values during winter in all three species. Qualitative microscopy also indicated species-specific erythrocyte morphological alterations. Most measured physicochemical variables were within the applicable environmental thresholds, whereas nitrate and nitrite nitrogen exceeded the regulatory values listed for moderate ecological status. As this is a preliminary study, contaminant concentrations were not measured in fish tissues. Moreover, since this study lacked an uncontaminated (reference) site, no causal relationship between water chemistry indices and hematological parameters could be established. However, the present findings provide baseline information on seasonal and interspecific variability in the hematological profiles of freshwater fishes, yet they require further analyses.

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1. Introduction

Considering that fish are highly sensitive to environmental changes and pollutants, they can serve as effective biological indicators of aquatic quality [1,2].

Blood is part of the internal environment of the organism, whose relative constancy and controlled variability within certain limits ensure optimal physicochemical conditions for the functioning of every cell [3]. Blood is also the first entry point for various anthropogenic pollutants through the gills and intestinal epithelial cells of fish, thereby affecting hematological characteristics, which are important indicators for evaluating cytotoxicity, environmental stress, and the health status of fish [4,5]. Furthermore, erythrocytes are among the formed elements of blood. Their number in peripheral circulation depends on multiple factors, including fish activity, water temperature, dissolved oxygen concentration, and other environmental variables, and it exhibits pronounced seasonal variability. In addition, erythrocyte counts are influenced by age, sex, nutritional status, and reproductive condition, and may also vary among populations of the same species [6]. The erythrocyte contains approximately 60% water and 40% dry matter, of which hemoglobin (Hb) is the most important component, accounting for about 90% of the dry mass [7]. Moreover, hemoglobin in vertebrates is an iron-containing complex protein located in red blood cells, responsible for oxygen transport throughout the body and for carrying carbon dioxide to the respiratory organs [8]. Nearly all vertebrates require a specialized oxygen transport system because molecular oxygen is poorly soluble in water: only 3.2 mL O_2 dissolves in 1 L of blood plasma. Thus, hemoglobin (Hb) can bind approximately 70 times more oxygen, reaching up to 220 mL O_2 per liter. Fish are frequently exposed to spatial and temporal fluctuations in oxygen availability and have therefore evolved anatomical, physiological, and biochemical strategies to adapt to changing environmental conditions [9]. Aerobic metabolism and the fulfillment of oxygen demand in fish are possible due to proteins, such as hemoglobin, which facilitate the transport of large amounts of oxygen to tissues, where it serves as the final electron acceptor [10]. Hb function appears to be adapted to the varying metabolic demands of fish and to continuous environmental changes [11,12]. Hematological parameters are affected by the animal's diet, environmental stress, or toxic substances. Hematological parameters can be used as reliable indicators of physiological changes [13]. According to Burgos-Aceves et al. [14], hematological indices serve as valuable prognostic and diagnostic tools for assessing the physiological well-being of fish. Furthermore, hematological studies of fish species in the marine sector are deemed to be an effective tool for predicting the host–environment relationship and subsequent migration of pollution onto higher predators up to top consumers [15–17].

That is why, with the present study, we aimed to conduct seasonal assessments of hematological parameters of the red blood profile in three freshwater fish species—common carp (*Cyprinus carpio*), Prussian carp (*Carassius gibelio*), and European perch (*Perca fluviatilis*)—inhabiting the Aleksandar Stamboliyski Reservoir (Bulgaria), in relation to key physicochemical characteristics of the aquatic environment. The hematological parameters included erythrocyte count (RBC, 10^{12}/L), hemoglobin concentration (Hb, g/dL), and hematocrit (PCV, %), as well as microscopic characterization of erythrocyte morphology. The water parameters included temperature, pH, dissolved oxygen concentration, electrical conductivity, and biochemical oxygen demand (BOD_5), as well as measured levels of

pollutants, such as nitrates, nitrites, total phosphorus, orthophosphates, Kjeldahl nitrogen, cadmium (Cd), chromium (Cr), cobalt (Co), lead (Pb), and zinc (Zn).

2. Materials and Methods

2.1. Study Area and Water Sampling

This study was conducted in the area of the Aleksandar Stamboliyski Reservoir (site code VTR-17208-1064, Executive Agency for Metrological and Technical Supervision, Bulgaria; BG1YN400L009; river basin code BG1000; EURBDCode BG1YN), located at coordinates 43°07′34.39″ N, 25°10′15.05″. The Aleksandar Stamboliyski Reservoir belongs to the Danube River Basin Directorate and is situated along the middle course of the Rositsa River, a tributary of the Yantra River. It collects water from the upper part of the Rositsa catchment, including inflows from the Krapets, Magara, and Karakaynak rivers. The catchment area upstream of the dam is approximately 1478 km^2 [18]. The reservoir is the largest in the Rositsa River basin. It was constructed on the Rositsa River and commissioned in 1953. The nearest settlement is the village of Gorsko Kosovo. The dam is a stone masonry structure with a reinforced concrete facing, with a height of 66 m and a crest length of 300 m. The total storage capacity is 205.6 × 10^6 m^3, including a dead storage of 20 × 10^6 m^3 and a useful storage of 185.6 × 10^6 m^3. The elevation at maximum water level is 190 m above sea level. The maximum surface area of the reservoir is 10.86 km^2, and the total catchment area is 1478 km^2. Downstream of the reservoir is the Rositsa-1 hydropower plant, with an installed capacity of 7.5 MW and a maximum discharge of 25 m^3/s, through which all released water passes. Further along the irrigation canals are the Rositsa-2 and Rositsa-3 hydropower plants, with installed capacities of 3 MW (Qmax = 12 m^3/s) and 0.28 MW (Qmax = 6 m^3/s), respectively. The reservoir supplies water to the Rositsa irrigation system, which covers a maximum area of approximately 27.000 ha [19].

This study covered the period from 2022 (based on data from the Danube River Basin Directorate, Bulgaria) through 2023 (own experimental investigations) and continued until February 2024. Water samples for physicochemical analysis (temperature, pH, and electrical conductivity) were measured in situ at five different sampling sites within the Aleksandar Stamboliyski Reservoir across the four seasons: spring (March–May), summer (June–August), autumn (September–November), and winter (December–February), using a combined pH meter (HANNA Instruments Combo HI98130, Smithfield, RI, USA).

2.2. Water Chemical Analyses

Water samples for chemical analysis were collected near the dam wall (GPS coordinates: 43°07′34.39″ N, 25°10′15.05″ E), in accordance with Directive 2008/105/EC [20] and the Bulgarian Regulation on environmental quality standards for priority substances and other pollutants. The analytical results were expressed as mean values based on 10 samples collected from five different sampling points. Samples were collected in clean, labeled polyethylene containers, transported on the same day, and stored at an appropriate temperature of 5 °C until processing at the external accredited laboratory for chemical analyses. It should be noted that water-quality parameters were determined at a single, fixed sampling location and are reported as monthly and seasonal mean values characterizing the reservoir at that point; they were not measured at the exact sites and times of fish capture and therefore do not represent individual-level exposure of the sampled fish. In the absence of paired individual water-chemistry and hematological data, the water-quality results are used as environmental context for the reservoir conditions during the study period rather than as exposure metrics for individual fish. The analyses of water pollutants (nitrates, nitrate nitrogen, nitrites, nitrite nitrogen, total phosphorus, orthophosphates, Kjeldahl nitrogen, Cr, Cd, Co, Pb, and Zn) were performed in accordance with EU requirements for

testing and calibration competence. All analyses were conducted using validated laboratory methods (VLM), in compliance with the following standards: BDS ISO 7890-3:1998, BDS EN 26777:1997, BDS EN ISO 6878:2005, BDS EN 1899-2:2004, BDS EN 25663:2002, BDS EN ISO 11885:2009, and BDS EN 25813:2004 [21–27]. Measurement uncertainty was expressed as expanded uncertainty, calculated as the standard measurement uncertainty multiplied by a coverage factor $k = 2$, corresponding to a confidence level of approximately 95% under normal distribution conditions.

2.3. Fish Sampling and Hematological Analyses

Common carp is considered one of the earliest domesticated fish species [28] and remains among the five most produced fish species in aquaculture worldwide [29]. It is farmed throughout its native range, with production concentrated primarily in Eastern Europe and far Eastern countries. Common carp is also among the most important freshwater aquaculture species worldwide, valued for its adaptability and resilience across diverse aquatic environments [30]. It is widely farmed and plays an important role in local economies and food security, with global production reaching about 5.1 million metric tons per year [31]. Common carp is also capable of bioconcentrating and bioaccumulating pollutants from water and sediments, which makes it a suitable sentinel organism. Its sensitivity, resilience to pollutants, and ease of laboratory maintenance have led to its frequent use as a bioindicator in toxicity testing and risk assessment [32].

Prussian carp is an important freshwater aquaculture species in China, recognized for its rapid growth, resilience to stress, and nutritional value. Production exceeded 2.70 million tons in 2021, corresponding to an economic value of about USD 5 billion [33]. Owing to its sensitivity to environmental pollutants, the species is also widely employed in ecotoxicology studies [34].

One fish species that is increasingly recognized as a promising candidate for the diversification of freshwater aquaculture is the European perch [35]. Eurostat [36] reported that 2022 marked the first recorded EU-level production of freshwater percid aquaculture, represented by pike-perch (*Sander lucioperca* (Linnaeus, 1758)) and European perch, with a combined output of 2.75 t live weight worth about EUR 30.000. Perch also plays a key role in shaping ecosystems [37] by exerting top-down ecological controls on food webs [38,39] and suppressing populations of invasive fish species [40]. In addition, in Sweden and Finland, the European perch is a primary sentinel species used by national environmental monitoring programs to measure aquatic toxicity, mercury (Hg) bioaccumulation, and "forever chemical" contamination [41].

The mean biometric characteristics were as follows: common carp—mean length $\mu = 35.65$ cm and mean weight $\mu = 878$ g (SD: 1.2658–17.7763; SE: 0.4003–5.6213); Prussian carp—mean length $\mu = 20.59$ cm and mean weight $\mu = 312.3$ g (SD: 0.8117–10.1788; SE: 0.2566–3.2188); European perch—mean length $\mu = 15.59$ cm and mean weight $\mu = 52.19$ g (SD: 0.6315–3.7063; SE: 0.1997–1.1720). For each species, 10 individuals per season were collected from the reservoir for analysis. To ensure comparability, fish of similar age and size groups were selected. Sampling was conducted in accordance with the Bulgarian Fisheries and Aquaculture Act [42]. Following measurement of total length and body weight, blood sampling was performed immediately after capture from visibly healthy and uninjured fish to avoid alterations in hematological parameters. Blood was collected once per individual from the caudal vein. To prevent coagulation, EDTA was used at a final concentration of 0.5%. Blood smears were prepared immediately after sampling. After blood collection, fish were released back into the reservoir. Collected blood samples were subjected to hematological analysis immediately after capture. The hematological profile was determined following established methodologies applied in fish and veterinary hema-

tology [15,43], as well as classical methods described by Dobrev et al. [3] and Ibrishimov and Lalov [44]. For erythrocyte counts (RBC), blood samples were appropriately diluted. Counts were performed in duplicate for each sample using a Bürker counting chamber. The chamber, thoroughly cleaned and dried, was fitted with a coverslip and placed under a microscope. After discarding the first drops from the pipette, a small volume of diluted blood was introduced at the edge of the coverslip, allowing capillary action to fill the chamber. Care was taken to avoid air bubble formation. After allowing 2–3 min for cell sedimentation, erythrocytes were counted under uniform distribution conditions. For erythrocyte counts (RBC), blood samples were appropriately diluted 1:200 and counted using a Bürker counting chamber in 80 small squares, according to the standardized fish hematology methodology described by Svobodova et al. [45]. Counting was performed in both chambers, and the mean value was used for calculations. The erythrocyte count per mm^3 was calculated using the following formula:

$$X = \frac{N}{80} \times 4000 \times 200 = N \times 10,000$$

where: X is the number of erythrocytes per mm^3, and N is the total number of counted erythrocytes in 80 small squares.

We performed morphometric analyses of erythrocytes on blood smears stained with a rapid staining kit (DKK "Color 200", VIVA-MT, Plovdiv, Bulgaria). Blood samples for hemoglobin determination were stored in VACUETTE® tubes (3 mL, 3.2% sodium citrate ($Na_3C_6H_5O_7$)). Hemoglobin concentration was measured using an EKF Diagnostics Hemo Control analyzer, based on direct photometric determination. Hematocrit (PCV, %) was determined by the microhematocrit method after centrifuging EDTA-anticoagulated blood in capillary tubes at 12,000 rpm for 5 min. Blood smears were examined under 40× and 100× objectives using Olympus CX22LEDRFS1 (Olympus Corporation, Tokyo, Japan) and Leica DM 500 (Leica Microsystems GmbH, Wetzlar, Germany) microscopes. Microphotographs were obtained using a Samsung Galaxy A21s (48 MP) (Samsung Electronics Co., Ltd., Suwon, Republic of Korea) camera. The following hematological parameters were determined: erythrocyte count (RBC); hemoglobin concentration (Hb); hematocrit (PCV). Additionally, microscopic characterization of erythrocyte morphology was performed. The present study includes a morphological characterization of blood cell findings (such as micronuclei, amitotic divisions, and poikilocytosis) as a complementary aspect, without attempting to link their frequency to environmental changes or pollution. Blood smears were stained using a differential rapid staining kit (DKK-COLOR/Diff-Quik type) to evaluate erythrocyte morphology. Microscopic images were captured and quantified using digital scale bars (μm) embedded directly into the figures to ensure accurate size visualization, rather than relying on total magnification descriptions.

2.4. Statistical Analyses

Statistical analyses were conducted using ANOVA and Tukey's HSD test in R (Statistical Computing). Calculations were also performed using Microsoft Excel. A significance level of $p < 0.05$ was used in all analyses. The following statistical parameters were used: x_i: individual value; $\overline{x}$: sample mean; s: sample standard deviation; σ: standard deviation; N: sample size; and μ: expected (mean) value. The percentage of altered blood cells was calculated as follows:

$$X\% = \frac{Y}{N} \times 100$$

where: $X\%$ is the percentage of altered cells, Y is the number of altered cells, and N is the total number of cells counted. The resulting data were converted into per mille (‰) by multiplying the outcome by 10. Experimental data are presented as mean values

($\sum / N \pm s^2$) for all examined individuals. All blood samples were analyzed in duplicate from the same specimen to ensure the reliability of the results.

3. Results and Discussion

3.1. Water Quality Assessment

During the study period, abiotic environmental factors were regularly monitored in the study area, and the physicochemical parameters of the water were recorded. The water temperature values for the Aleksandar Stamboliyski Reservoir are presented in Table 1.

Table 1. Temperature measurements in surface water samples from the Aleksandar Stamboliyski Reservoir (n = 10 measurements per month).

Month, Year	Aleksandar Stamboliyski Reservoir GPS Coordinates: 43°07′34.39″ N, 25°10′15.05″ E		
	T °C		
	Range (Min–Max)	Arithmetic Mean	Standard Deviation
III, 2023	6–8	6.8	0.79
IV, 2023	10–14	11.8	1.48
V, 2023	16–21	18.16	1.53
VI, 2023	19–21	20.3	0.67
VII, 2023	20–25	22	1.41
VIII, 2023	21–25	22.9	1.29
IX, 2023	21–24	22.53	1.17
X, 2023	19–22	20.1	0.88
XI, 2023	12–15	13.34	1.38
XII, 2023	5–15.7	7.37	3.02
I, 2024	4–6	5.15	0.58
II, 2024	4–6	5.2	0.59

The recorded temperature values showed the following patterns: the mean temperatures in March, April, and May were 6.80, 11.80, and 18.6 °C, respectively. The mean temperature in June was 20.30 °C, followed by 22.0 °C in July and 22.90 °C in August. In September, the average recorded temperature was 22.53 °C, decreasing to 20.10 °C in October, and a sharp decline to 12.0 °C was observed in November. During the winter season, temperatures decreased further to approximately 4 °C; the mean values were 7.37 °C in December, 5.15 °C in January, and 5.20 °C in February. The minimum and maximum recorded water temperatures in the Aleksandar Stamboliyski Reservoir during the study period ranged as follows: March–May, 6–21 °C; June–August, 19–25 °C; September–November, 12–24 °C; and December–February, 4–15.7 °C. Increasing temperature increases metabolic rate and oxygen consumption. When the temperature deviates above or below optimal limits, organisms slow down and may eventually cease vital functions. The thermal optimum for most fish species, such as salmon, sturgeon, perch, and carp, lies within the ranges of 13–18, 20–24, 22–28, and 24–30 °C, respectively. The upper limits of vital activity for these species are approximately 23–28, 31–35, 32–37, and 34–41 °C, respectively [46,47].

The values of the active reaction (pH) of the water in the Aleksandar Stamboliyski Reservoir measured during the study period are presented in Table 2.

Table 2. pH measurements in surface water samples from the Aleksandar Stamboliyski Reservoir (n = 10 measurements per month).

Month, Year	Aleksandar Stamboliyski Reservoir GPS Coordinates: 43°07′34.39″ N, 25°10′15.05″ E		
	pH		
	Range (Min–Max)	Arithmetic Mean	Standard Deviation
III, 2023	7.2–7.8	7.51	0.20
IV, 2023	7.5–8	7.77	0.19
V, 2023	7.2–7.9	7.71	0.24
VI, 2023	7.9–8	7.94	0.05
VII, 2023	7.6–8.5	8.02	0.30
VIII, 2023	7.7–8.1	7.99	0.12
IX, 2023	7.5–8.1	7.91	0.16
X, 2023	8–8.2	8.08	0.09
XI, 2023	8–8.5	8.32	0.18
XII, 2023	8.7–9.2	8.89	0.16
I, 2024	8–8.7	8.43	0.23
II, 2024	7.9–8.2	8.02	0.09

The results showed that the mean pH values were 7.51 in March, 7.77 in April, and 7.71 in May. In June, July, and August, the mean values were 7.94, 8.02, and 7.99, respectively. In September, the mean pH was 7.91, increasing to 8.08 in October and 8.32 in November. During the winter season, higher pH values were recorded, with mean values of 8.89 in December, 8.43 in January, and 8.02 in February. The minimum and maximum recorded pH values ranged as follows: March–May, 7.2–8.0; June–August, 7.0–8.5; September–November, 7.5–8.5; and December–February, 7.9–9.2. Higher pH values were generally observed during the winter season. pH is of critical importance for fish adaptation to changing environmental conditions [48].

The electrical conductivity of the water in the Aleksandar Stamboliyski Reservoir measured during the study period is presented in Table 3.

Table 3. Conductivity measurements in surface water samples from the Aleksandar Stamboliyski Reservoir (n = 10 measurements per month).

Month, Year	Aleksandar Stamboliyski Reservoir GPS Coordinates: 43°07′34.39″ N, 25°10′15.05″ E		
	Conductivity (µS/cm)		
	Range (Min–Max)	Arithmetic Mean	Standard Deviation
III, 2023	305–310	307.9	2.51
IV, 2023	310–315	311.9	2.28
V, 2023	302–310	306.1	3.00
VI, 2023	309–312	310.4	1.43
VII, 2023	308–310	308.6	0.70
VIII, 2023	321–330	327.8	3.61
IX, 2023	315–320	318.5	1.58
X, 2023	318–320	319.3	0.95
XI, 2023	320–335	330	5.77
XII, 2023	340–348	345.6	2.55
I, 2024	335–340	336.5	2.42
II, 2024	320–336	330.7	4.62

The mean conductivity values were 307.9 µS/cm in March, 311.9 µS/cm in April, and 306.1 µS/cm in May. In June, July, and August, the values were 310.4, 308.6, and 327.8 µS/cm, respectively. In September, October, and November, the mean values were 318.5, 319.3, and 330.0 µS/cm, respectively. An increase in conductivity was observed during the winter season, with mean values of 345.6 µS/cm in December, 336.5 µS/cm in January, and 330.7 µS/cm in February. The minimum and maximum recorded conductivity values ranged as follows: March–May, 302–315 µS/cm; June–August, 308–330 µS/cm; September–November, 315–335 µS/cm; and December–February, 320–348 µS/cm. According to Regulation H-4, conductivity values below 650 µS/cm are classified as excellent.

Data from physicochemical monitoring of the waters of the Aleksandar Stamboliyski Reservoir, based on a report by the Danube River Basin Directorate under the Ministry of Environment and Water of Bulgaria (request No. 01-69/2024), including BOD_5 and dissolved oxygen concentrations in surface waters, are presented in Table 4.

Table 4. Physicochemical monitoring of surface waters from the Aleksandar Stamboliyski Reservoir, based on data provided by the Danube River Basin Directorate, Pleven, Bulgaria.

Monitoring Location—Code	Water Body—Code	Date	BOD_5 (mg/L)	Dissolved Oxygen (mg/L)
BG1YN43199MS021	BG1YN400L1009	24 January 2022	1.34 *	8.72 *
BG1YN43199MS021	BG1YN400L1009	22 March 2022	1.5 *	13 *
BG1YN43199MS021	BG1YN400L1009	27 April 2022	0.5 *	9.02 *
BG1YN43199MS021	BG1YN400L1009	17 January 2024	7.83 ± 0.34	9.17 ± 0.40

* Historical monitoring data provided by the Danube River Basin Directorate, Pleven, Bulgaria. Values obtained in the present study are presented as mean ± SD (n = 10 water samples).

The monitoring results indicated that dissolved oxygen levels were within the legal thresholds and classified as excellent. For BOD_5, values exceeding 2.5 mg/L indicated a moderate ecological status during the winter period of 2024 (7.83 ± 0.34), according to Regulation H-4 [49] (Table 5).

Table 5. Physico-chemical quality elements in the context of the Water Framework Directive, WFD 2000/60/EC. Values are presented as mean ± standard deviation (SD), based on 10 water samples collected from five sampling sites (two samples per site).

Ecological Status Classification	Dissolved Oxygen (mg/L)	pH	Conductivity (µS/cm)	BOD_5 (mg/L)
Excellent	10.5–8.00	-	<650	<1
Good	8.00–6.00	6.5–8.7	750	1–2.5
Moderate	<6.00	-	>750	>2.5

It has been established that under hypoxic conditions, many Amazonian fish obtain oxygen directly from the air due to anatomical modifications, including changes in the gills, mouth, stomach, and intestines [50,51]. Species lacking this ability rely on alternative physiological mechanisms, such as increased ventilation rate and volume, elevated heart rate, increased erythrocyte counts, higher hematocrit and hemoglobin concentrations, changes in organic phosphate levels, the presence of hemoglobin isoforms with different functional properties, and metabolic [50]. In some cases, fish may acclimate to hypoxia. According to Pan et al. [52], acclimation to hypoxia is not associated with changes in gill morphology, hematocrit, or relative ventricular mass. Their findings support the hypothesis that switching hemoglobin isoforms may provide a physiological advantage in coping with environmental stress. Some Cyprinids, such as goldfish (*Carassius auratus* (Linnaeus, 1758)),

are capable of tolerating low oxygen levels, temperature fluctuations, and high levels of anthropogenic pollution; therefore, they are frequently used as model or control species in laboratory studies [53–55].

The analyses of water quality for orthophosphate, nitrate nitrogen ($N–NO_3^-$), and nitrite nitrogen ($N–NO_2^-$) are presented in Table 6 and compared according to Regulation H-4 (Table 7).

Table 6. Chemical measurements (nutrients) in surface water samples from the Aleksandar Stamboliyski Reservoir.

№	Chemical Substance	Unit of Measurement	Results (Average ± St. Deviation)
1.	NO_3^-	mg/L	23.00 ± 1.05
2.	NO_2^-	mg/L	0.24 ± 0.006
3.	$N\text{-}NO_3^-$	mg/L	5.198
4.	$N\text{-}NO_2^-$	mg/L	0.0729
5.	P and—P—ortho-PO_4	mg/L	0.60 ± 0.06

Table 7. Chemical quality elements in the context of the Water Framework Directive, WFD 2000/60/EC.

Ecological Status Classification	P—Ortho—PO_4 (mg/L)	$N\text{-}NO_3$ (mg/L)	$N\text{-}NO_2$ (mg/L)
Excellent	<0.008	<0.2	<0.01
Good	0.008–0.016	0.2–0.5	0.01–0.025
Moderate	>0.016	>0.5	>0.025

Laboratory analyses revealed elevated concentrations of nitrate nitrogen ($N–NO_3^-$) and nitrite nitrogen ($N–NO_2^-$) in the waters of the Aleksandar Stamboliyski Reservoir. The levels of nitrates and nitrites in water are of critical importance for maintaining good ecological status and the health of aquatic biota. Nitrites negatively affect the chemical and hydrobiological characteristics of water, ultimately impacting aquatic organisms. For example, sodium nitrite at initial concentrations of 0.25 mg/L has been shown to reduce dissolved oxygen levels in water [56]. Furthermore, low oxygen levels or oxygen depletion may alter the balance between nitrification and denitrification processes within the ecosystem [57]. The primary toxic effect of nitrates on aquatic animals is associated with the conversion of oxygen-carrying pigments (hemoglobin, hemocyanin) into forms incapable of transporting oxygen (methemoglobin) [58–61]. Absorbed nitrite reacts with hemoglobin to form methemoglobin, which in adult organisms is rapidly reduced back to oxyhemoglobin by enzymatic systems such as NADH-dependent methemoglobin reductase. In aquaculture systems, toxic concentrations of nitrites may arise due to high stocking densities and intensive feeding practices. In natural water bodies, elevated nitrite levels are typically associated with wastewater contamination. Prolonged exposure to nitrites induces oxidative stress in fish [62]. A relevant example is the exceedance of permissible limits for nitrogen (ammonium and nitrite), total phosphorus, and orthophosphates by 6- to 20-fold in water samples from the Cherna River (Smolyan), collected following a fish mortality event involving approximately 160 kg of fish on 20 August 2023. These data were reported by the Basin Directorate–Plovdiv [63].

Table 8 presents analyses of toxic metal concentrations in the waters of the Aleksandar Stamboliyski Reservoir and compares them with the maximum permissible concentrations (MPCs) set by Directive (EU) 2008/105 [20]. With the exception of cadmium (see below), the measured concentrations of toxic metals were within permissible limits according to both European Directives and Bulgarian legislation.

Table 8. Chemical measurements (metals) in surface water samples from the Aleksandar Stamboliyski Reservoir.

№	Toxic Metal	Unit of Measurement	Results (Average ± St. Deviation)	DIRECTIVE 2008/105/EC on Environmental Quality Standards in the Field of Water Policy, MAC-EQS-Inland Surface Waters
1.	Chrome (Cr)	µg/L	1.30	-
2.	Cobalt (Co)	µg/L	0.60 ± 0.002	-
3.	Zinc (Zn)	mg/L	<0.001	-
4.	Cadmium (Cd)	mg/L	<0.02	≤0.45 (Class 1) 0.45 (Class 2) 0.6 (Class 3) 0.9 (Class 4) 1.5 (Class 5)
5.	Lead (Pb)	µg/L	8.50 ± 0.17	Not applicable

Regarding Pb, the World Health Organization recommends maintaining the current parametric value while emphasizing that concentrations should be reduced as much as reasonably achievable. Accordingly, the value of 10 µg/L should be maintained for 15 years following the entry into force of Directive (EU) 2020/2184 [64], after which it should be reduced to 5 µg/L. Due to the continued presence of Pb pipes in buildings and the limited authority of Member States to enforce their replacement, the 5 µg/L threshold remains a target value for domestic distribution systems. However, for all new materials in contact with drinking water, a limit of 5 µg/L should be applied (Directive (EU) 2020/2184) [64]. The chemical analyses indicated Cd concentrations reported as <0.02 mg/L (<20 µg/L), i.e., below the limit of quantification of the analytical method used. It is important to emphasize that this quantification limit (≈20 µg/L) is higher than the Cd parametric value of 5 µg/L; consequently, although Cd was not detected above 20 µg/L, compliance with the 5 µg/L standard cannot be confirmed from the present data, and Cd should not be reported as conclusively "within permissible limits." Cd is classified as a priority hazardous substance under Directive 2000/60/EC [65]. The environmental quality standards for Cd and its compounds (No. 6) vary depending on water hardness, categorized into five classes: class 1: <40 mg $CaCO_3$/L; class 2: 40 to <50 mg $CaCO_3$/L; class 3: 50 to <100 mg $CaCO_3$/L; class 4: 100 to <200 mg $CaCO_3$/L; and class 5: ≥200 mg $CaCO_3$/L. Because the present analysis could not verify Cd concentrations below the 5 µg/L standard, confirmation of Cd compliance with the relevant environmental quality standards will require re-analysis using a more sensitive method, such as inductively coupled plasma mass spectrometry (ICP-MS). The following general considerations on metal toxicity are provided as background context and are not intended to imply that metal intoxication was responsible for the observed hematological changes. Contamination of aquatic ecosystems with toxic metals can lead to reductions in key hematological parameters in fish, most commonly hemoglobin (Hb), erythrocyte count (RBC), and packed cell volume (PCV), and in some cases mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). For example, exposure to Cr and copper (Cu) has been associated with increased MCV, while Cd exposure affects MCH [66]. In the present study, the measured concentrations of the analyzed toxic metals (Cr, Co, Pb, and Zn) were within the permissible limits set by the relevant European Directives and Bulgarian legislation; the only exception is Cd, for which compliance with the 5 µg/L standard could not be confirmed because the analytical quantification limit (≈20 µg/L) exceeded the standard. Metal intoxication is therefore not indicated as a driver of the observed hematological changes for those metals whose compliance was verified. In contrast, the contribution of Cd cannot be excluded based on the present data and remains to be assessed with a more sensitive analytical method. For accurate interpretation of results, it is essential to

consider a range of variables, including reproductive cycle, age, sex, feeding behavior, stress, nutritional status, water quality, and species habitat, as poikilothermic organisms are strongly influenced by environmental changes [14].

3.2. Hematological Assessment

The hematological parameters of the red blood profile of the studied fish species are presented in Table 9.

Table 9. Hematological parameters of the red blood profile of the studied fish species from the Aleksandar Stamboliyski Reservoir.

Common carp	**Season**							
	Spring		**Summer**		**Autumn**		**Winter**	
	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$
RBC ($\times 10^{12}$/L)	1.311	0.0100	1.325	0.0255	1.291	0.0255	1.317	0.0074
Tukey HSD test (RBC)	spring/summer ns; spring/autumn ns; spring/winter ns; summer/autumn ns; summer/winter ns; autumn/winter ns							
Hb (g/dL)	13.57	0.5524	11.95	0.6084	11.36	0.3519	13.85	0.4658
Tukey HSD test (Hb)	spring/summer ns; spring > autumn ($p = 0.028$); spring/winter ns; summer/autumn ns; summer/winter ns; autumn < winter ($p = 0.011$)							
PCV (%)	37.1	1.6938	33.2	1.8963	34.1	1.0435	39.85	1.5492
Tukey HSD test (PCV)	spring/summer ns; spring/autumn ns; spring/winter ns; summer/autumn ns; summer < winter ($p = 0.037$); autumn/winter ns							
Prussian carp	**Season**							
	Spring		**Summer**		**Autumn**		**Winter**	
	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$
RBC count ($\times 10^{12}$/L)	1.3	0.0667	0.877	0.0241	0.919	0.0166	1.322	0.0309
Tukey HSD test (RBC)	spring > summer ($p < 0.001$); spring > autumn ($p < 0.001$); spring/winter ns; summer/autumn ns; summer < winter ($p < 0.001$); autumn < winter ($p < 0.001$);							
Hb (g/dL)	7.35	0.4065	5.85	0.4060	6.47	0.3443	7.52	0.2869
Tukey HSD test (Hb)	spring > summer ($p = 0.043$); spring/autumn ns; spring/winter ns; summer/autumn ns; summer < winter ($p = 0.020$); autumn/winter ns							
PCV (%)	30	1.1489	22.12	0.2945	24.1	0.3301	31.8	0.7720
Tukey HSD test (PCV)	spring > summer ($p < 0.001$); spring > autumn ($p < 0.001$); spring/winter ns; summer/autumn ns; summer < winter ($p < 0.001$); autumn < winter ($p < 0.001$);							
European perch	**Season**							
	Spring		**Summer**		**Autumn**		**Winter**	
	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$	$\bar{x}$	$\sigma_{\bar{x}}$
RBC ($\times 10^{12}$/L)	1.298	0.0110	1.278	0.0378	1.073	0.0153	1.3	0.0108
Tukey HSD test (RBC)	spring/summer ns; spring > autumn ($p < 0.001$); spring/winter ns; summer > autumn ($p < 0.001$); summer/winter ns; autumn < winter ($p < 0.001$);							
Hb (g/dL)	11.65	0.5240	10.21	0.1252	9.98	0.2344	11.95	0.6084
Tukey HSD test (Hb)	spring/summer ns; spring/autumn ns; spring/winter ns; summer/autumn ns; summer < winter ($p = 0.043$); autumn < winter ($p = 0.018$)							
PCV (%)	30.9	0.8056	31.4	0.8390	27.65	1.1959	33.2	1.8963
Tukey HSD test (PCV)	spring/summer ns; spring/autumn ns; spring/winter ns; summer/autumn ns; summer/winter ns; autumn < winter ($p = 0.027$)							

Differences between means were tested for significance using a Tukey HSD test. *p*-values are presented for each comparison that passes the significance threshold ($p < 0.05$). Non-significant differences are denoted with "ns".

The results of hematological analyses in fish depend on numerous intrinsic and extrinsic factors, including stress levels [67], blood sampling methods [68], pre-analytical factors such as anticoagulant use [69], blood storage temperature and duration [43,70,71], and analytical procedures, including the type of diluent [72] and accuracy of blood cell classification.

The results obtained for erythrocyte counts in specimens inhabiting the Aleksandar Stamboliyski Reservoir follow this order: spring–common carp > Prussian carp > European perch; summer–common carp > European perch > Prussian carp; autumn–common carp > European perch > Prussian carp; winter–Prussian carp > common carp > European perch. The highest erythrocyte counts were recorded in common carp during spring, summer, and autumn, whereas in winter the highest values were observed in Prussian carp. During spring and winter, values for all three species were relatively similar. In most fish species, erythrocytes are oval, nucleated, and larger than those of mammals [73]. Their number typically ranges from 0.8×10^6 to $3.5 \times 10^6/mm^3$, while leukocyte counts vary between 20.000 and $50.000/mm^3$, reaching up to $100.000/mm^3$ in some species [74]. Erythropoiesis in vertebrate fish occurs primarily in the kidneys, with or without spleen involvement [75]. Generally, erythrocyte counts range from $0.5–1.5 \times 10^6/mm^3$ in less active species to $3.0–4.2 \times 10^6/mm^3$ in more active species [66]. Furthermore, fish erythrocytes are sensitive to environmental pollution, and their morphological evaluation can serve as a bioindicator of toxicity [66].

The observed pattern across all seasons was: common carp > European perch > Prussian carp. The highest hemoglobin concentrations for all three species were recorded during winter. Hemoglobin levels are influenced by sex, age, season, and environmental conditions [75]. Additionally, hemoglobin oxygen affinity varies seasonally, with lower affinity in summer and higher affinity in winter [76]. Similar seasonal variation has been reported in *Hoplosternum littorale* (Hancock, 1828) [77]. Variations in hemoglobin patterns related to temperature acclimation have also been observed in Goldfish, supporting the hypothesis of polymorphism as an adaptive mechanism [78].

Hematocrit values followed the pattern: common carp > European perch > Prussian carp across all seasons.

Hematological parameters are highly sensitive to environmental factors, including nutrition, water quality, stress, and pathogens. Peripheral blood tests include measurements of various erythrocyte and leukocyte indices and are often complemented by biochemical analyses [79]. Hematocrit values are strongly associated with the biological activity level of fish. Highly active species, such as tuna and other pelagic fish, typically exhibit higher hematocrit values than benthic species such as flatfish. Therefore, hematological parameters are relative, and clear distinctions between normal and abnormal values are often difficult to define [80].

The morphological characteristics of common carp, Prussian carp, and European perch are illustrated in Figures 1–3.

Fish erythrocytes are nucleated and typically exhibit a slightly elliptical shape. Alongside normal erythrocytes, cells with altered morphology were observed, including changes in both cell shape and nuclear structure. In common carp, erythrocytes with nuclear alterations were identified in addition to normal cells ($3.70 \pm 0.23‰$) (Figure 1). Normal erythrocytes displayed an elliptical, centrally located nucleus surrounded by homogeneous cytoplasm (Figure 1). In Prussian carp, microscopic examination revealed a considerable number of dividing cells, particularly during winter ($28.70 \pm 3.79‰$) (Figure 2). This is most plausibly interpreted as physiological preparation for the reproductive period in spring, especially as all examined individuals were female, although other seasonal factors, including water temperature and pH, may also play a role. Because pollutant

concentrations were not measured in fish tissues and no uncontaminated reference site was available, a causal contribution of the elevated winter nitrate and nitrite concentrations to the observed erythrocyte division cannot be inferred from the present data. A plausible explanation may be the physiological preparation of the organism for the spring reproductive period, combined with the low water temperatures. Since most European populations of Prussian carp consist predominantly of females reproducing through gynogenesis [81], this pronounced erythropoietic response appears to be characteristic of the female organism in this species. Our findings are fully consistent with the seasonal dynamics reported by Bojarski et al. [82], who documented a high number of dividing erythrocytes in Prussian carp, specifically during the winter season. The relationship between temperature fluctuations and erythrocyte modifications in this species was also previously demonstrated by Dekić et al. [83], while the ability of erythrocytes in the genus *Carassius* to undergo direct division in the peripheral circulation as an adaptive mechanism was described in detail by Murad et al. [84]. These findings confirm that the observed peak represents a normal seasonal adaptive response. In European perch, rounded erythrocytes (8.80 ± 0.18‰) and micronuclei (9.70 ± 0.23‰) were observed (Figure 3).

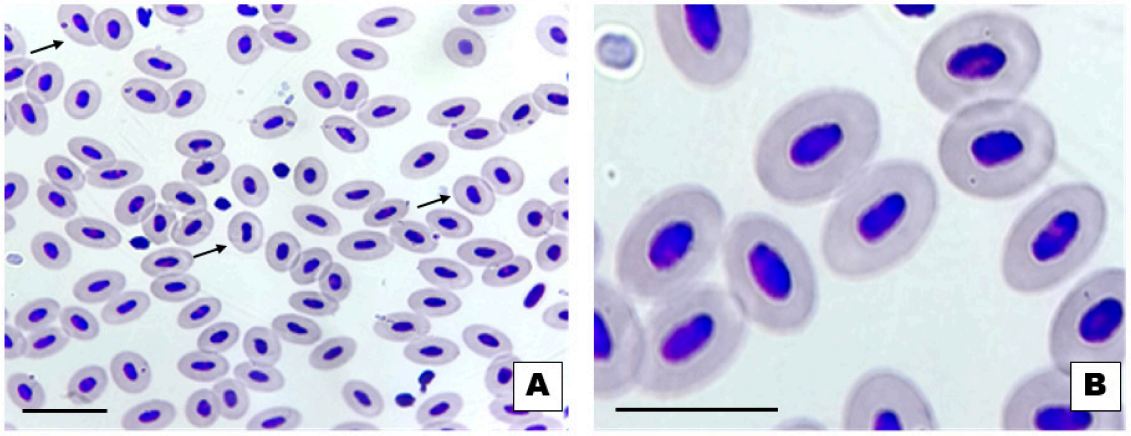


Figure 1. Red blood cells of common carp. (**A**) Erythrocytes with nuclear alterations (arrows). (**B**) Erythrocytes showing normal morphology, ×100, ×1000. Scale bars: (**A**) 100 µm; (**B**) 10 µm (DCC-COLOR/Diff-Quik type).

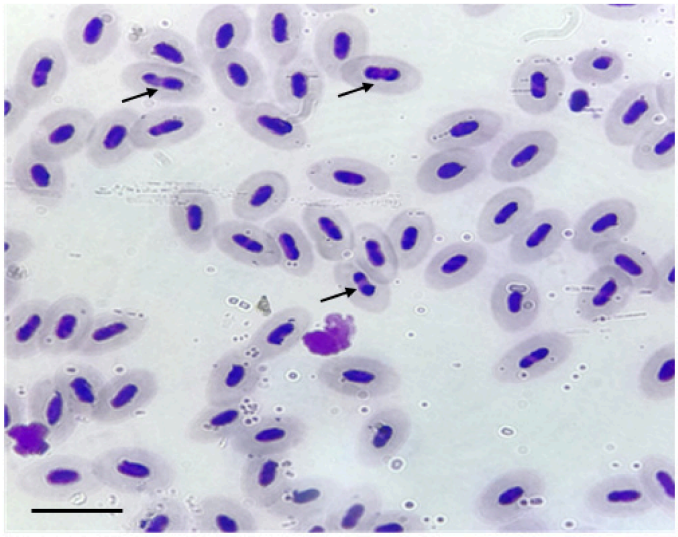


Figure 2. Dividing erythrocytes of Prussian carp (arrows), ×400. Scale bar: 25 µm (DCC-COLOR/Diff-Quik type).

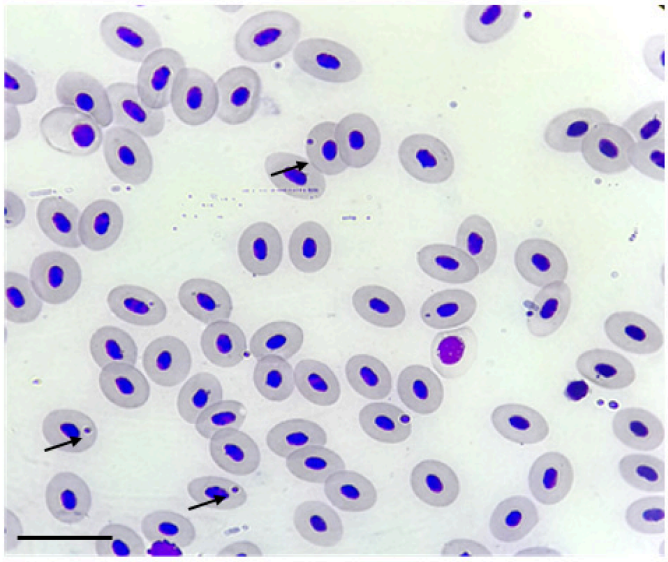


Figure 3. Morphological alterations in the erythrocytes of European perch, including rounded cells and cells with micronuclei (arrows), ×400 Scale bar: 25 µm (DCC-COLOR/Diff-Quik type).

Rounded erythrocytes may represent immature cells, as immature forms are more commonly found in fish blood compared to mammals. According to Cavas et al. [85], the frequency of micronuclei and binucleated cells increases significantly in fish inhabiting freshwater environments contaminated with low concentrations of copper. Hematopoietic organs in fish are primarily located in the intratubular tissues of the kidneys. In trout, the spleen is also active; in roach, only the kidney is active; whereas in perch, hematopoiesis occurs mainly in the spleen [86]. Similar morphological alterations in erythrocytes of freshwater fish exposed to metal mixtures have been reported by other authors, who consider such changes to be sensitive indicators of toxic metal intoxication [87]. As the toxic metal concentrations measured in the reservoir water were within permissible limits, however, the present morphological observations should not be attributed to metal intoxication; their association with the recorded environmental conditions remains to be tested in future studies using tissue-level contaminant analysis and reference-site comparisons. In addition, at low concentrations, fish may gradually adapt to metal exposure. The effects of toxic metals on hematological parameters depend on both concentration and exposure duration. In such cases, primarily morphological changes are observed, including an increased proportion of aged and disintegrated cells and amitotic erythrocytes. In contrast, erythrocyte counts, hemoglobin concentration, and hematocrit values may remain within normal ranges [88]. Hematological parameters are of fundamental importance for fish physiology, as their alteration serves as an indicator of functional imbalance or pathological conditions. These parameters may vary due to changes in feeding and reproductive status, stress, toxic exposure, infectious diseases, and the influence of abiotic environmental factors [89].

The results of statistical analyses using ANOVA and Tukey's HSD test are presented in Tables 10–14 and Figures 4–8. The one-way ANOVA on the hematological data for Prussian carp suggested that the season of capture significantly affected all examined parameters except MCHC: RBC, Hb, PCV, MCV, and MCH (parameters with statistically valid differences in mean values between at least two seasons). A Tukey HSD test was applied to evaluate between-group differences in the examined hematological parameters. In Prussian carp, RBC and PCV were significantly higher in spring and winter than in summer and autumn. Hb was lower in the summer compared to both spring and winter. Both MCV and MCH were lower in the spring compared to the autumn, while MCV alone was significantly lower in the winter than in the autumn (Table 1). The one-way ANOVA

on the hematological parameters for European perch revealed significant between-season differences in mean values for RBC, Hb, PCV, MCH, and MCHC but not for MCV (Table 2). The post hoc comparison (Tukey HSD test) revealed that RBC was significantly lower in the autumn than in any other season. Hb was significantly higher in the winter than in summer and autumn, while PCV was higher in the winter than in autumn alone. MCH appeared higher in the autumn than in the summer, while MCHC was higher in the spring than in the summer (Table 2). The analysis of hematological parameters for common carp suggested significant seasonal alterations for Hb, PCV, MCV, MCH, and MCHC but not for RBC. The post hoc evaluation of the observed mean differences demonstrated that Hb in common carp is lower in the autumn than in the spring and winter. PCV was significantly higher in the winter than in summer, while MCV was higher in the winter than in summer and autumn. MCH was higher in the spring and winter than in the summer and autumn, while MCHC was higher in the spring and winter compared to the autumn alone (Table 3). The Kaiser-Meyer-Olkin analysis indicated that the experimental data were probably suitable for factor analysis, with an overall score of 0.63. The PCA results for the Prussian carp hematological parameters suggested that the first three principal components explained 99% of the total variance (50.07, 38.48, and 11.27%, respectively). The same applies to the European perch data, where each of the first three principal components explains 47.49, 29.33, and 23.03% of the variance. The percentages of variance calculated for the first three principal components using the common carp parameters were 68.92, 19.16, and 11.88%, respectively. All calculated PC coefficients are summarized in Table 4. Bi-plot diagrams showing both the sample scores (as dots) and the variable loadings (as vectors) with reference to the first two main axes are presented in Figure 4. When discriminant analysis (DA) was applied to the Prussian carp data, it produced a set of functions that clearly separated the samples captured in the spring and winter from those captured in the summer and autumn. DA suggested that a set of four parameters with factor loadings greater than 0.7, PCV, MCH, MCV, and RBC, were able to correctly estimate the season of capture for Prussian carp (spring, summer, autumn or winter): PCV (Wilks' lambda = 0.247; F (36) = 36.5; $p < 0.001$), MCH (Wilks' lambda = 0.192; F (36) = 3.3; $p = 0.031$), MCV (Wilks' lambda = 0.140; F (36) = 4.2; $p = 0.012$), RBC (Wilks' lambda = 0.109; F (36) = 3.1; $p = 0.037$). The calculated squared Mahalanobis distances as measures of group separation were as follows: spring vs. summer: 18.582, spring vs. autumn: 20.680, spring vs. winter: 1.838, summer vs. autumn: 1.809, summer vs. winter: 25.596, autumn vs. winter: 27.138. The clustering of individual samples based on the spatial distributions of linear discriminant functions produced two clearly distinguishable clusters: the first one consisting of samples captured during the spring and winter and the second one that includes samples captured during the summer and autumn (Figure 5A). Two of the examined hematological parameters showed a negative correlation with the first discriminant function (LD1): RBC (−0.65) and PCV (−0.68). In contrast, the parameter MCH (0.30) exhibited a positive correlation with LD1. Taken together, the other two discriminant functions, LD2 and LD3, explain less than 7% of the observed variation in the dataset (Table 5). The mean values of LD1 for Prussian carp individuals for each season were calculated as follows: spring (LD1: −1.981), summer (LD1: 2.252), autumn (LD1: 2.446), winter (LD1: −2.717). Generally, the constructed DA model exhibits classification potential significantly higher than random classification by chance alone: Cohen's kappa = 0.63 (CI: 0.45–0.82). The discriminant analysis (DA) of hematological data for European perch obtained results quite similar to those for Prussian carp. The constructed DA model revealed that as few as two parameters (possessing factor loadings greater than 0.7), RBC and MCHC, could account for the variation in the dataset: RBC (Wilks' lambda = 0.344; F (36) = 22.8; $p < 0.001$), MCHC (Wilks' lambda = 0.273; F (36) = 3.0; $p = 0.043$). The squared Mahalanobis distances

calculated with the European perch data for each season were as follows: spring vs. summer: 4.757, spring vs. autumn: 38.760, spring vs. winter: 0.770, summer vs. autumn: 23.407, summer vs. winter: 3.527, autumn vs. winter: 35.807. Comparably to that of Prussian carp, the clustering of individual European perch samples showed all spring and winter samples visibly separated from summer and autumn samples (Figure 5B). The correlation analysis determined one of the examined parameters as negatively correlated with the first discriminant function (LD1): RBC (−0.51), while the other parameters, Hb (−0.19), PCV (−0.17), MCV (0.06), MCH (0.05), and MCHC (0.00), exhibited weak or no correlation with LD1. Taken together, the other two discriminant functions, LD2 and LD3, explain less than 7% of the observed variation in the dataset (Table 5). The mean values of LD1 for every season obtained for the European perch data were as follows: spring (−1.996), summer (−0.461), autumn (4.212), winter (−1.755). Generally, the constructed DA model exhibits classification potential significantly higher than random classification by chance alone: Cohen's kappa = 0.63 (CI: 0.45–0.82). The results of the discriminant analysis of the common carp data suggested that a set of three parameters, MCH, MCV, and MCHC, were sufficient to correctly discriminate the individual samples from the species based on the season of capture: MCH (Wilks' lambda = 0.589; F (36) = 8.3; $p < 0.001$), MCV (Wilks' lambda = 0.331; F (36) = 9.1; $p < 0.001$), MCHC (Wilks' lambda = 0.280; F (36) = 2.0; $p = 0.128$). The seasonal separation of individual samples by the squared Mahalanobis distances was measured as follows: spring vs. summer: 5.215, spring vs. autumn: 9.476, spring vs. winter: 3.169, summer vs. autumn: 4.960, summer vs. winter: 5.975, autumn vs. winter: 4.985. The clustering of individual samples based on the constructed model is presented in Figure 5C. Three of the examined hematological parameters exhibited a strong negative correlation with the first discriminant function (LD1): Hb (−0.50), MCH (−0.61), and MCHC (−0.56), while the parameters RBC (−0.09), PCV (−0.28), and MCV (−0.31) demonstrated weak negative correlations with LD1. Meanwhile, MCHC (−0.54) was the only parameter to show a strong negative correlation with the second canonical function, LD2, while PCV (0.42), MCV (0.59), and MCH (0.43) exhibited a positive correlation with LD2. Taken together, the first two discriminant functions, LD1 and LD2, explain more than 90% of the observed variation in the dataset (Table 5). The mean values of LD1 and LD2 for each season based on the common carp data were as follows: spring (LD1: −1.463, LD2: 0.136), summer (LD1: 0.352, LD2: 1.309), autumn (LD1: 1.556, LD2: −0.452), winter (LD1: −0.445, LD2: −0.993). The calculated Cohen's kappa indicated that the constructed DA model had a classification performance higher than random chance: Cohen's kappa = 0.60 (CI: 0.42–0.78). Table 5 presents the obtained coefficients of linear discriminants for each parameter of interest.

Table 10. One-way ANOVA: tests of between-season differences (A_{df}: Season, B_{df}: Residuals; MS: Mean square) for hematological parameters in common carp (n = 10 fish per species per season).

	RBC	Hb	PCV	MCV	MCH	MCHC
A_3: MS	0.002	14.788	91.56	5324	802.6	22.385
F (3.36)	0.51 ns	5.23 **	3.31 *	5.30 **	8.35 ***	8.18 ***
B_{36}: MS	0.004	2.823	27.65	1003	96.1	2.733
Tukey HSD test	1/2 ns; 1/3 ns; 1/4 ns; 2/3 ns; 2/4 ns; 3/4 ns;	1/2 ns; 1 > 3 *; 1/4 ns; 2/3 ns; 2/4 ns; 3 < 4 *;	1/2 ns; 1/3 ns; 1/4 ns; 2/3 ns; 2 < 4 *; 3/4 ns;	1/2 ns; 1/3 ns; 1/4 ns; 2/3 ns; 2 < 4 **; 3 < 4 *;	1 > 2 *; 1 > 3 **; 1/4 ns; 2/3 ns; 2 < 4 **; 3 < 4 **;	1/2 ns; 1 > 3 ***; 1/4 ns; 2 > 3 **; 2/4 ns; 3/4 ns;

Legend: RBC: erythrocyte count, Hb: hemoglobin concentration, PCV: packed cell volume (hematocrit value), MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration. Significance codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$. Tukey HSD test: 1: spring; 2: summer; 3: autumn; 4: winter. Significance codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$.

Table 11. One-way ANOVA: tests of between-season differences (A_{df}: Season, B_{df}: Residuals; MS: Mean square) for hematological parameters in Prussian carp.

	RBC	Hb	PCV	MCV	MCH	MCHC
A_3: MS	0.572	6.108	214.21	1772	451.0	24.01
F (3.36)	32.88 ***	4.14 *	36.52 ***	3.67 *	7.17 ***	1.28 ns
B_{36}: MS	0.017	1.475	5.87	482	62.9	18.70
Tukey HSD test	1 > 2 ***; 1 > 3 ***; 1/4 ns; 2/3 ns; 2 < 4 ***; 3 < 4 ***;	1 > 2 *; 1/3 ns; 1/4 ns; 2/3 ns; 2 < 4 *; 3/4 ns;	1 > 2 ***; 1 > 3 ***; 1/4 ns; 2/3 ns; 2 < 4 ***; 3 < 4 ***;	1/2 ns; 1 < 3 *; 1/4 ns; 2/3 ns; 2/4 ns; 3/4 ns;	1/2 ns; 1 < 3 **; 1/4 ns; 2/3 ns; 2/4 ns; 3 > 4 **;	1/2 ns; 1/3 ns; 1/4 ns; 2/3 ns; 2/4 ns; 3/4 ns;

Legend: RBC: erythrocyte count, Hb: hemoglobin concentration, PCV: packed cell volume (hematocrit value), MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration. Significance codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$. Tukey HSD test: 1: spring; 2: summer; 3: autumn; 4: winter. Significance codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$.

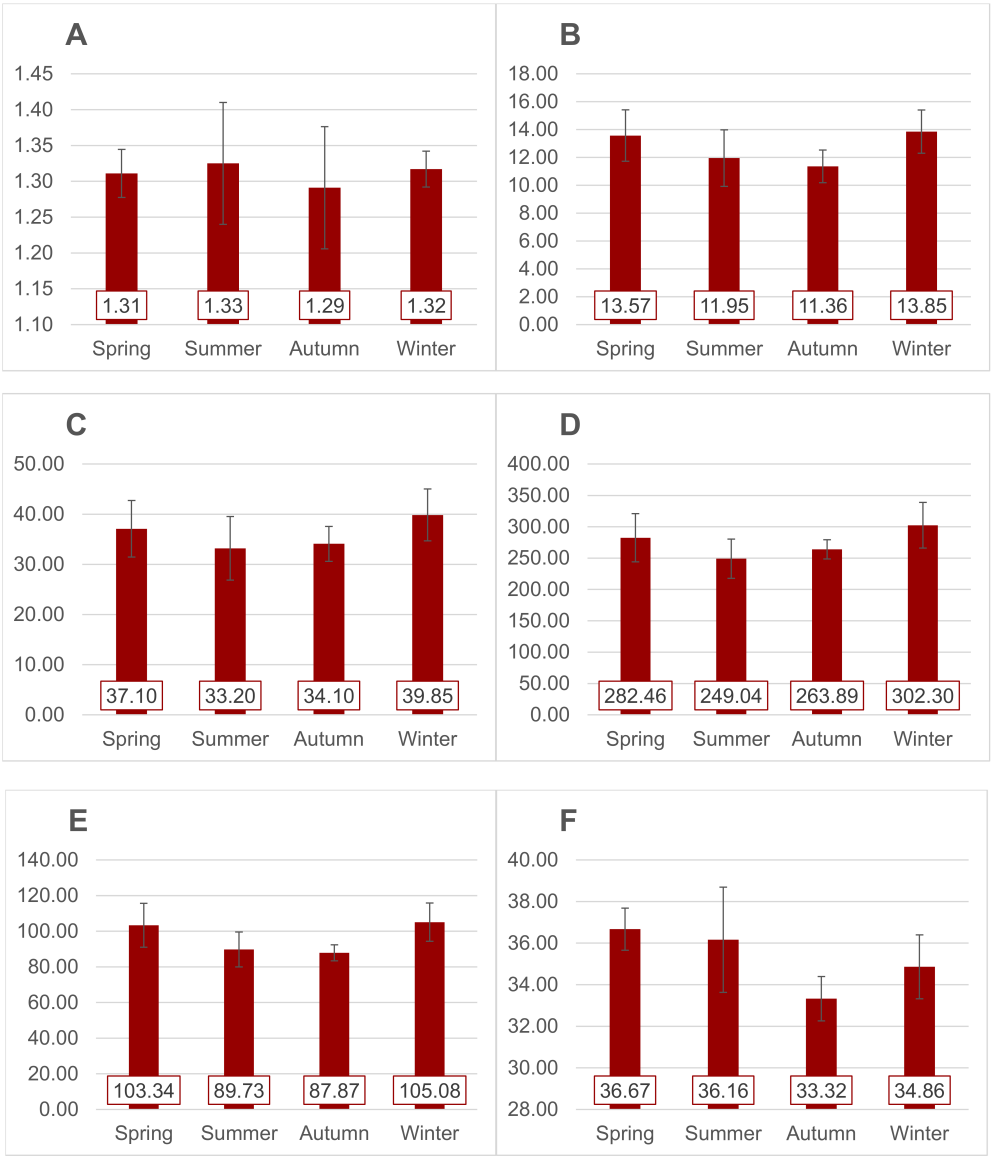


Figure 4. Seasonal dynamics of hematological parameters (mean ± SD) in common carp (**A**): RBC, (**B**): Hb, (**C**): PCV, (**D**): MCV, (**E**): MCH; (**F**): MCHC.

Table 12. One-way ANOVA: tests of between-season differences (A_{df}: Season, B_{df}: Residuals; MS: Mean square) for hematological parameters in European perch.

	RBC	Hb	PCV	MCV	MCH	MCHC
A_3: MS	0.120	9.928	53.51	748.5	305.19	44.86
F (3.36)	22.84 ***	4.99 **	3.02 *	0.75 ns	3.08 *	3.16 *
B_{36}: MS	0.005	1.988	17.72	986.8	99.03	14.16
Tukey HSD test	1/2 ns; 1 > 3 ***; 1/4 ns; 2 > 3 ***; 2/4 ns; 3 < 4 ***;	1/2 ns; 1/3 ns; 1/4 ns; 2/3 ns; 2 < 4 *; 3 < 4 *;	1/2 ns; 1/3 ns; 1/4 ns; 2/3 ns; 2/4 ns; 3 < 4 *;	1/2 ns; 1/3 ns; 1/4 ns; 2/3 ns; 2/4 ns; 3/4 ns;	1/2 ns; 1/3 ns; 1/4 ns; 2 < 3 *; 2/4 ns; 3/4 ns;	1 > 2 *; 1/3 ns; 1/4 ns; 2/3 ns; 2/4 ns; 3/4 ns;

Legend: RBC: erythrocyte count, Hb: hemoglobin concentration, PCV: packed cell volume (hematocrit value), MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration. Significance codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$. Tukey HSD test: 1: spring; 2: summer; 3: autumn; 4: winter. Significance codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$.

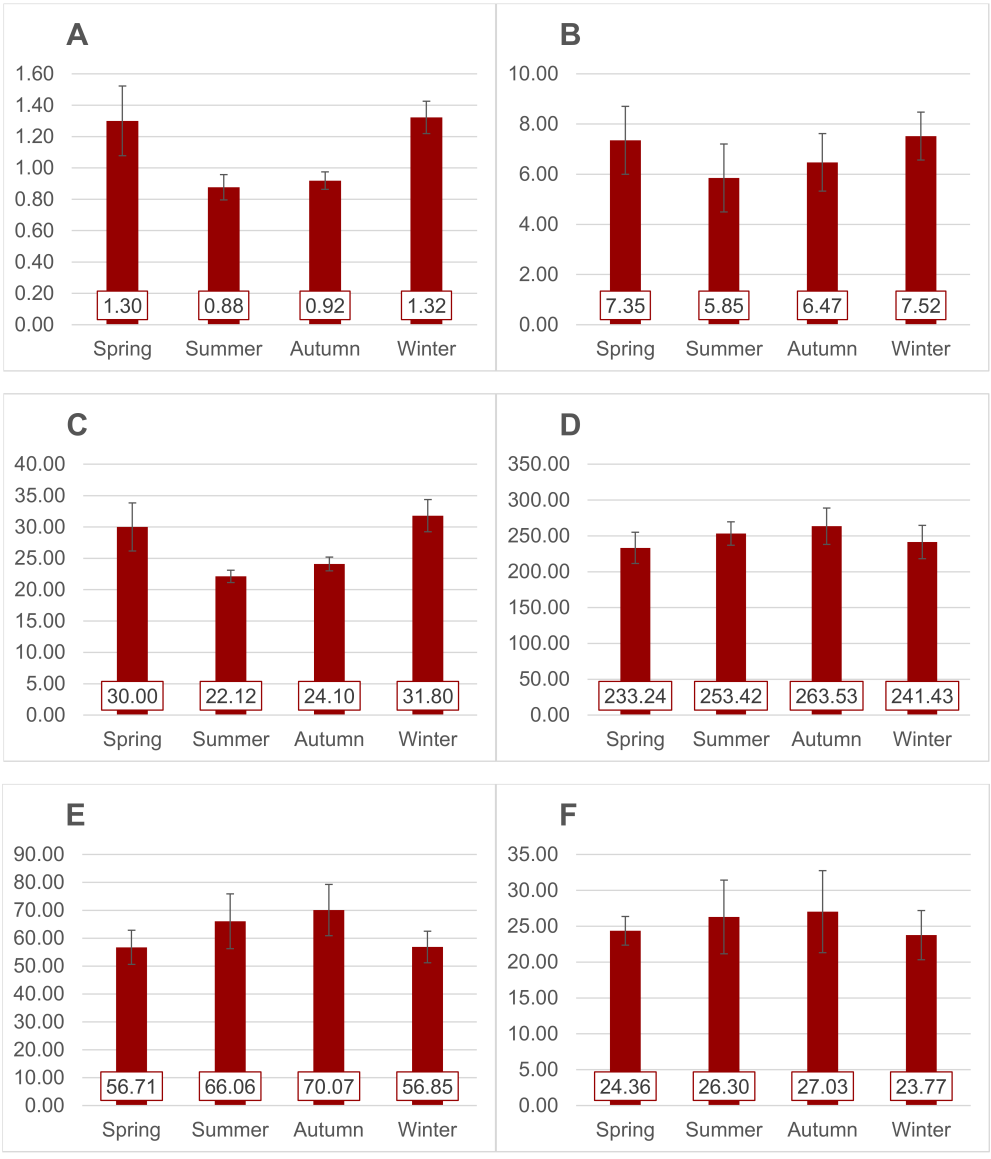


Figure 5. Seasonal dynamics of hematological parameters (mean ± SD) in Prussian carp (**A**): RBC, (**B**): Hb, (**C**): PCV, (**D**): MCV, (**E**): MCH; (**F**): MCHC.

Table 13. Principal component coefficients and percentages of variance in the examined hematological parameters.

	Common Carp		PRUSSIAN CARP		European Perch	
Parameter	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
RBC	0.316	0.209	0.560	−0.155	0.194	−0.183
Hb	0.482	0.185	0.500	0.283	0.523	0.272
PCV	0.487	−0.126	0.476	−0.273	0.534	−0.309
MCV	0.464	−0.226	−0.426	−0.176	0.457	−0.249
MCH	0.461	0.151	−0.121	0.614	0.440	0.435
MCHC	−0.064	0.912	0.116	0.643	−0.026	0.739
Percentage of Variance	68.92%	19.16%	50.07%	38.48%	47.49%	29.33%
Eigenvalue	4.13	1.15	3.00	2.31	2.85	1.76

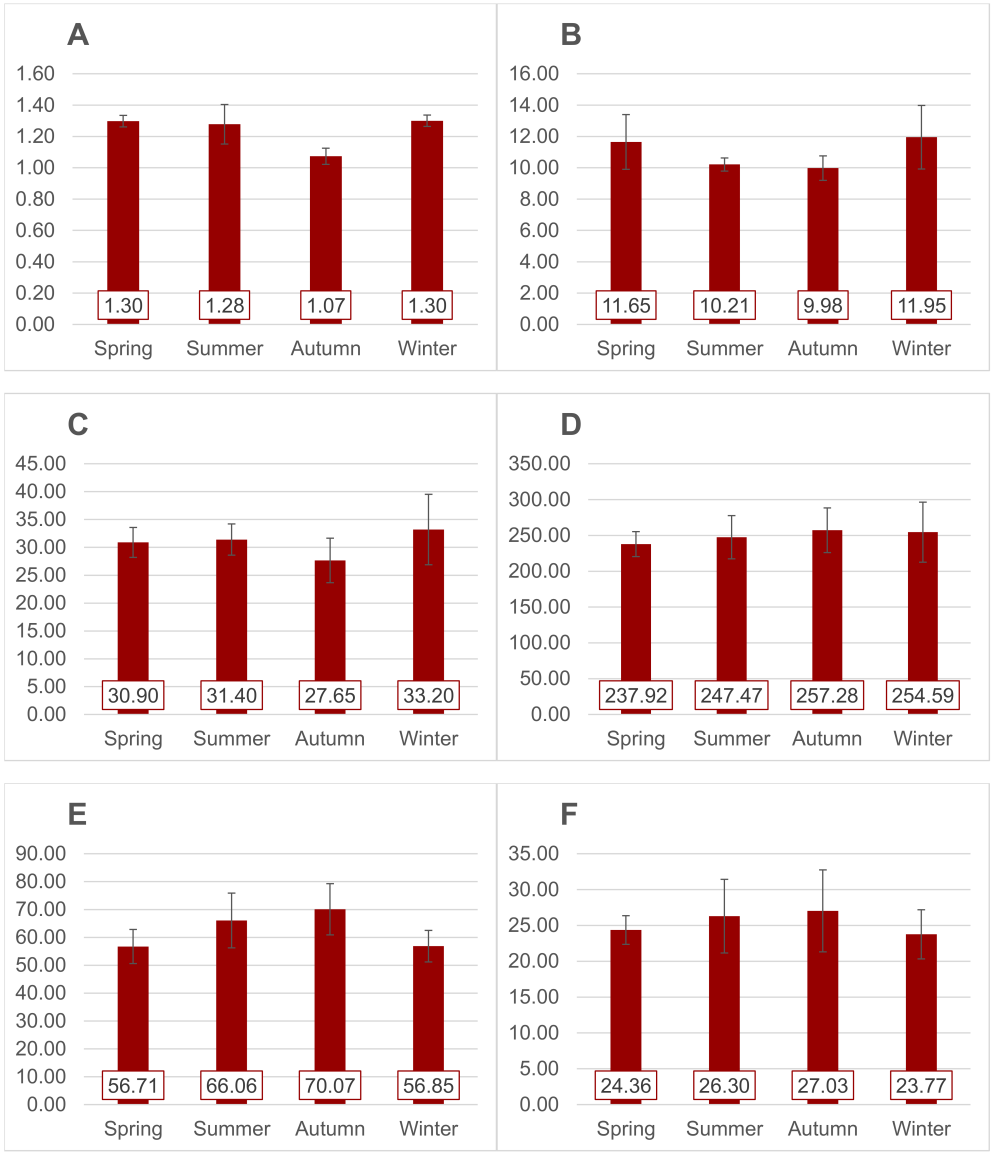
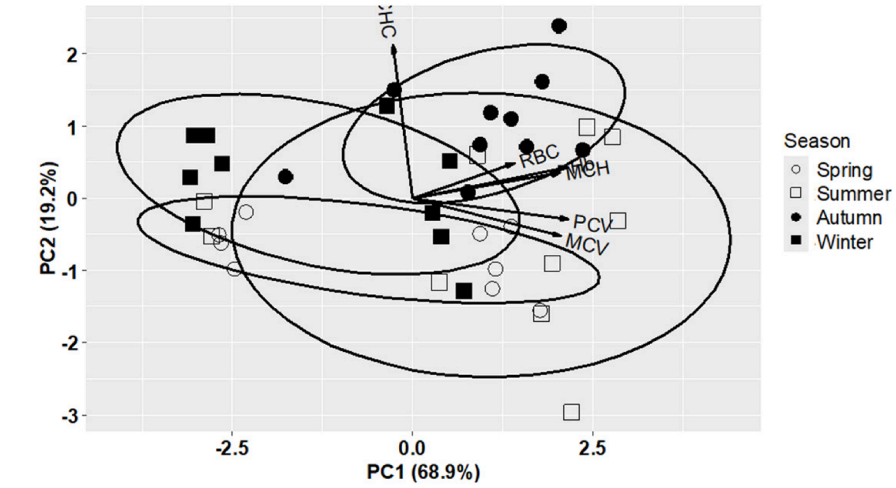
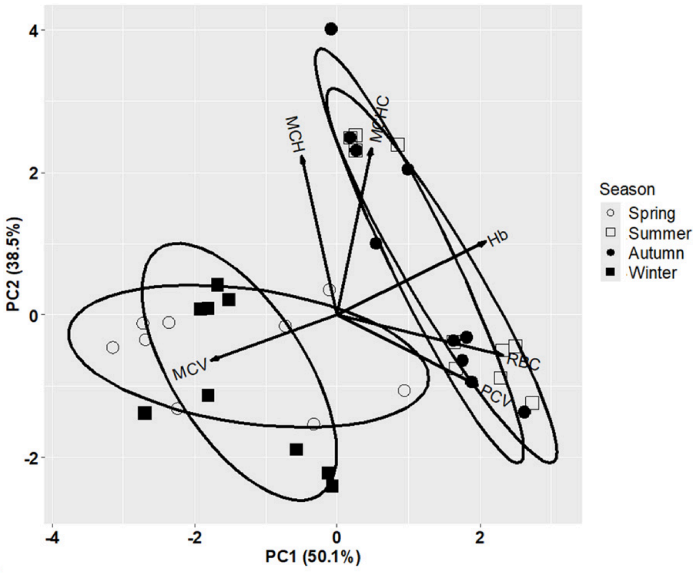


Figure 6. Seasonal dynamics of hematological parameters (mean ± SD) in European perch (**A**): RBC, (**B**): Hb, (**C**): PCV, (**D**): MCV, (**E**): MCH; (**F**): MCHC.



A



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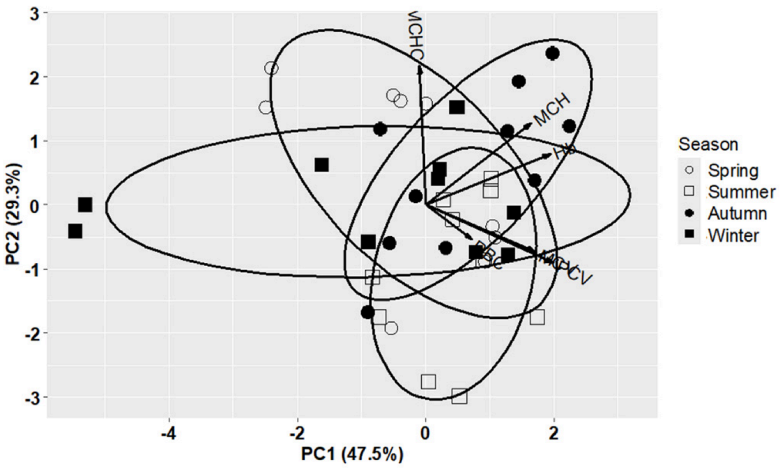

C

Figure 7. PCA of the examined hematological data. (**A**) Common carp, (**B**) Prussian carp, (**C**) European perch.

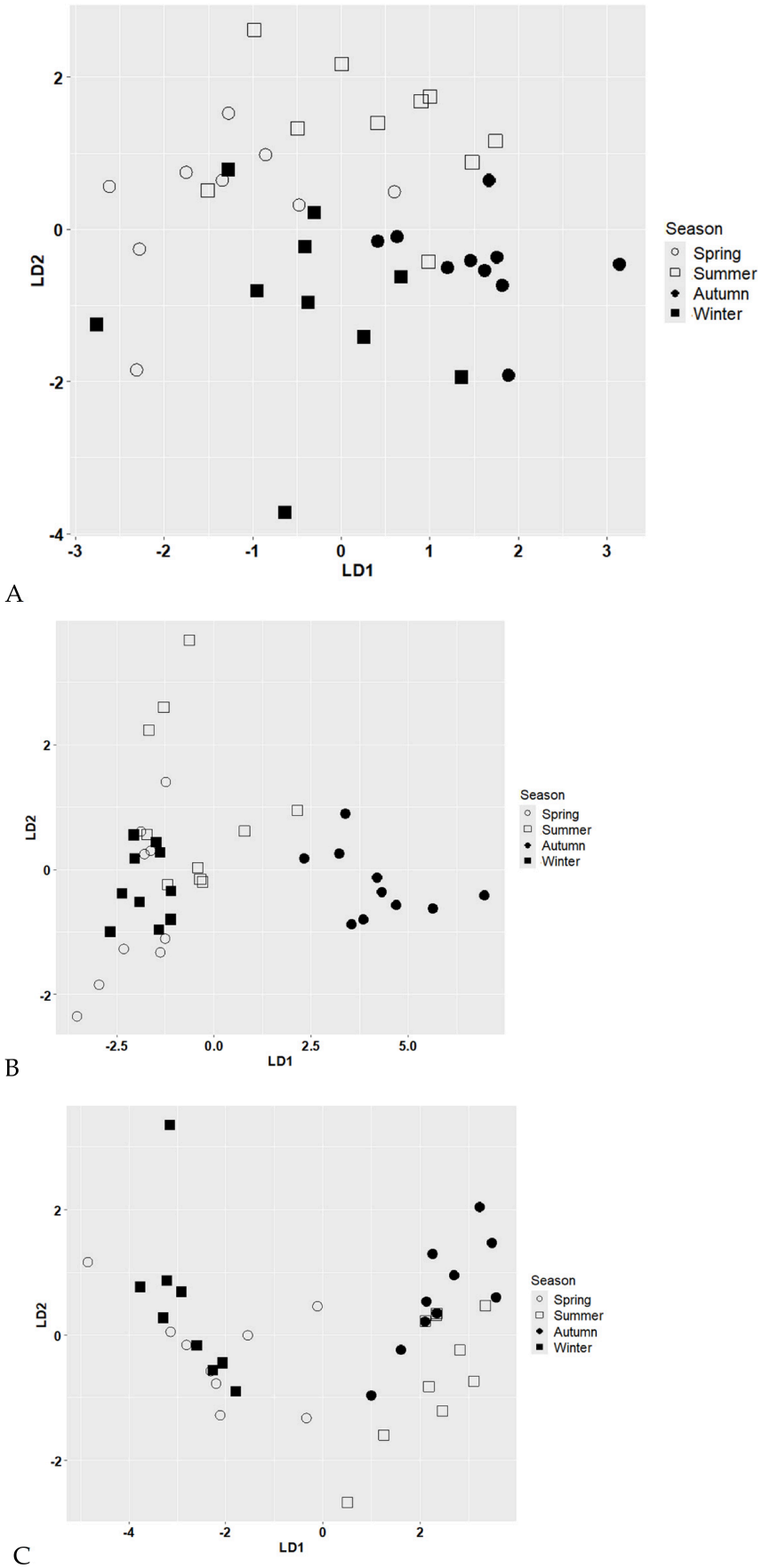

Figure 8. Clustering of individual samples based on the obtained linear discriminants. (**A**) Common carp, (**B**) Prussian carp, (**C**) European perch.

Table 14. Coefficients of linear discriminants and proportions of the trace of discriminant models constructed with the examined hematological parameters (data in brackets represent standardized coefficients).

	Common Carp			Prussian Carp			European Perch		
Parameter	**LD1**	**LD2**	**LD3**	**LD1**	**LD2**	**LD3**	**LD1**	**LD2**	**LD3**
RBC	16.053 (1.023)	135.890 (8.661)	−67.950 (−4.331)	29.563 (3.900)	18.390 (2.426)	44.601 (5.884)	79.038 (5.750)	18.585 (1.352)	−33.174 (−2.413)
Hb	8.682 (14.588)	−12.132 (−20.385)	−0.203 (−0.340)	−1.805 (−2.192)	−3.218 (−3.909)	0.634 (0.770)	−13.486 (−19.013)	−3.485 (−4.913)	−2.884 (−4.066)
PCV	−3.369 (−17.714)	−0.965 (−5.075)	2.599 (13.667)	−1.140 (−2.761)	0.220 (0.534)	−1.889 (−4.574)	0.858 (3.613)	0.540 (2.272)	2.396 (10.086)
MCV	0.661 (20.948)	0.195 (6.166)	0.186 (5.886)	0.099 (2.178)	0.151 (3.308)	−0.015 (−0.320)	−0.064 (−2.000)	−0.022 (−0.698)	−0.313 (−9.835)
MCH	−1.853 (−18.161)	1.467 (14.384)	−1.444 (−14.151)	0.364 (2.888)	−0.111 (−0.877)	0.971 (7.699)	1.683 (16.752)	0.284 (2.829)	0.469 (4.663)
MCHC	1.187 (1.962)	0.022 (0.036))	3.826 (6.325)	−0.184 (−0.796)	1.270 (5.493)	−2.389 (−10.332)	0.261 (0.982)	0.137 (0.517)	−0.284 (−1.068)
Proportion of trace	0.578	0.346	0.075	0.935	0.042	0.022	0.935	0.052	0.012
Eigenvalue	1.357	0.812	0.176	6.214	0.280	0.147	6.951	0.389	0.091

4. Conclusions

The results of the present study indicate that hematological parameters, including erythrocyte count, hemoglobin concentration, and hematocrit, vary with season, with the highest values generally recorded during winter, and that these seasonal dynamics differ among species. The highest hematological values were observed in common carp, followed by European perch and Prussian carp; these differences may be attributed to species-specific variations in physiological activity, feeding behavior, and ecological characteristics. Species-specific alterations in erythrocyte morphology were identified in all three studied species. However, because contaminant concentrations were not measured in fish tissues, this study lacked an uncontaminated reference site, and as no statistical exposure–response analysis was performed, these morphological alterations cannot be attributed to nitrate, nitrite, or any other measured pollutant. They are more cautiously interpreted as seasonal hematological variation occurring concurrently with the recorded physicochemical conditions, recognizing that hematological parameters in fish are additionally influenced by factors that the present design could not control for, including water temperature, dissolved oxygen, reproductive condition, sex, age, nutritional status, and handling. Elevated concentrations of nitrate and nitrite nitrogen were detected in the reservoir water, exceeding the reference values for moderate ecological status; although these concentrations indicate nutrient enrichment of the reservoir and warrant continued monitoring, they cannot be linked causally to the observed hematological changes on the basis of the present data. The use of artificial fertilizers should nonetheless be carefully managed, as nutrient leaching through groundwater may contribute to reservoir contamination and pose a potential threat to the aquatic ecosystem. A limitation of the present study is the absence of data on pollutant bioaccumulation in fish tissues, the lack of an uncontaminated reference site, and the absence of a statistical exposure–response analysis; together, these constraints preclude the establishment of a causal relationship between contaminant exposure and the observed hematological alterations. In addition, the water-quality parameters were measured at a single, fixed sampling location and are reported as monthly and seasonal means, whereas

the fish hematological data were obtained from discrete seasonal samples; the two datasets therefore differ in spatial and temporal resolution and were not paired at the individual level; so, the water-quality results should be regarded as environmental context rather than as individual-level fish exposure. This study could also not fully account for non-pollutant factors known to influence hematological parameters, including water temperature, dissolved oxygen, reproductive condition, sex, age, nutritional status, and handling stress. Finally, for Cd, the analytical quantification limit (≈20 µg/L) exceeded the relevant 5 µg/L standard; so, compliance with the Cd environmental quality standard could not be verified. Future studies should, therefore, measure contaminant concentrations in fish tissues (gills, liver, and kidneys), include one or more uncontaminated reference sites, pair water-quality and biological sampling in space and time, apply exposure–response analyses, and use sufficiently sensitive analytical methods (e.g., ICP-MS) for trace elements such as Cd while controlling for the principal biological and environmental confounders to better elucidate the link between environmental pollution and fish health. Yet, this preliminary study provides information on the seasonal and interspecific variability in hematological parameters in three freshwater fish species from the Aleksandar Stamboliyski Reservoir and represents a useful baseline for future, more mechanistic investigations.

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Institutional Review Board Statement: Ethical review and approval were not obtained for this study. The fish were captured during the legally open fishing season under valid fishing permits issued by the Executive Agency for Fisheries and Aquaculture (IARA), Bulgaria. This study involved catch-and-release sampling of common carp, Prussian carp, and European perch. The fish were anesthetized, blood samples were collected from the caudal vein, and all individuals were subsequently released alive at the sites of capture. No fish were sacrificed or retained, and no threatened or protected species were involved. All procedures were designed to minimize handling time, stress, and potential injury to the animals.

Data Availability Statement: The original contributions presented in this study are included in this article. Further inquiries can be directed to the corresponding author(s).

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