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HEMATOLOGICAL CHARACTERISTICS OF *DELMINICHTHYS GHETALDII* (STEINDACHNER 1882) INHABITING THE KARST REGION OF EASTERN HERZEGOVINA

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Abstract - Hematological parameters are a valuable means of estimating the physiological status of fish and, indirectly, the state of their habitat. In order to use blood parameters as biomarkers, it is necessary to know their normal values and the referential intervals for a species. Such investigations are especially valuable in endemic and endangered fish species. In this study, parameters of erythrocyte lineage of *Delminichthys ghetaldii* (Steindachner 1882), an endemic species inhabiting the karst waters of eastern Herzegovina, were investigated. Fish were caught by nets in the region of Fatničko polje (Fatnica field) after the water outflow in March 2013, blood was taken by heart puncture and erythrocyte parameters were determined. Their mean values were as follows: the number of erythrocyte (RBC) = 1.474 x 10^{12}/l; hemoglobin concentration (Hb) = 72.50 g/l; packed cell volume (PCV) = 0.398 l/l; mean corpuscular volume (MCV) = 271.19 fl; mean corpuscular hemoglobin (MCH) = 49.36 pg, and mean corpuscular hemoglobin concentration (MCHC) = 193.16 g/l eryt. Body mass and morphometric features were also estimated and their mean values were: body mass = 25.49 g, total and standard body length = 14.13 cm and 11.98 cm, respectively, Fulton coefficient = 1.45. For the values of all parameters, the normality of distribution was tested, as well as differences between mean values of males and females. Pearson's correlation and multiple regression analyses between mass, morphometric and hematological parameters have shown that erythrocyte parameters may influence body mass and of morphometric characteristics in this species.

Key words: *Delminichthys ghetaldii*; erythrocyte lineage; biomarkers

INTRODUCTION

Study of the hematological features of wild fish is extremely important, especially if we consider that natural waters are exposed to different anthropogenic activities. These actions mostly encompass water utilization for various purposes, thereby altering the ecosystem. These alterations result in changes in the basic ecological factors of an aquatic environment. Physiological and biochemical fish characteristics are reliable indicators of the condition of their organism as well as the condition of the population and habitat in time and space (Ivanc and Dekić, 2012). The prerequisite for using these data is knowledge of the physiological range of their variations.

The hematological status covers the erythroid and myelin lineages and has a high diagnostic value for the definition of phylogenetically specified physiological features of a species, as well as the habitat-

conditioned physiological characteristics. The parameters of erythrocyte and leukocyte lineages are also relevant indicators of fish health, and the estimated values for a given species help understand the degree of the variation during different phases of the life cycle as well as an estimation of the normal values typical of the species (Ivanc and Miljanović, 2003).

Alterations in the blood parameters appear with reference to age, sex, reproductive cycle phase, nutrition specificities, and the complex of ecological factors of the habitat (Vázquez and Guerrero, 2007). Ichthyological and hematological research is very important, especially for endemic species that occupy limited areas. The endemic fish species of our target area are largely connected with the undergound waters of karst regions.

The karst regions of Eastern Herzegovina are interesting for several reasons. They have a specific hydrology due to large underground water-filled caves with surface abysses from which periodically emerge plunging streams that flood the fields. These waters are inhabited by the endemic fish species *Delminichthys ghetaldii* (Steindachner 1882) and *Telestes metohiensis* (Steindachner, 1901), which are on the red list ("Official Gazette of the Republic of Srpska", No. 142/12). Data on the biological and physiological features of these species are poor and fragmented, which makes new data extremely pertinent.

The previous studies on *Delminichthys ghetaldii* in Eastern Herzegovina were mostly based on distribution (Vuković, 1971; Kottelat and Freyhof, 2007; Dekić et al., 2011), morphometric features (Bogutskaya et al., 2012), and only partly on hematological research (Dekić et al., 2012). Therefore, the aim of this paper is to collect and interpret the basic hematological data of the *Delminichthys ghetaldii,* an endemic species that occurs massively in the Fatničko polje (Fatnica field) during the flooding season.

MATERIALS AND METHODS

The research was performed on *Delminichthys ghetaldii* specimens that were caught in the Fatničko polje area after its flooding. The flooding is connected to weather conditions and occurs periodically, mostly in spring and autumn after heavy precipitation. At the same time, the water breaks from the upper abyss part of the cave, bringing to the surface fish that spend part of their lifetime cycle in underground waters.

According to an old taxonomy, the *Delminichthys ghetaldii* of this area belongs to the genus *Paraphoxinus*, which included the following species: *Paraphoxinus metohiensis* (Steindachner, 1901), *Paraphoxinus pstrossi* (Steindachner, 1882), and *Paraphoxinus ghetaldii* (Steindachner 1882). Subsequently, they were included in a new *Phoxynellus* genus with the following species (Dekić et al., 2011): *Phoxynellus metohiensis* (Steindachner, 1901), *Phoxynellus pstrossi* (Steindachner, 1882) and *Phoxynellus ghetaldii* (Steindachner, 1882).

According to recent taxonomy, fish from the genus *Phoxynellus* are divided into two genera: *Telestes* and *Delminichthys* (Freyhof et al., 2006; Kottelat and Freyhof, 2007).

Bogutskaya et al. (2012) claims that there are morphological variations within the *Telestes metohiensis* species and though once thought to be a single species, should now be divided into three separate species: the existing *Telestes metohiensis* and two new ones, i.e. *Telestes dabar* (Bogutskaya, Zupančić, Bogut & Naseka, 2012) and *Telestes miloradi* (Bogutskaya, Zupančić, Bogut & Naseka, 2012).

Site

The research area is located in Eastern Herzegovina, i.e. the southeastern part of Republic of Srpska and Bosnia and Herzegovina. Regarding relief evolution and the recent regional morphology, the primary factors are the stratigraphic and lithostratigraphic content of Eastern Herzegovina. Most of our target area (Dabarsko and Fatničko polje – Dabar and Fatnica fields) is a typical Dinarides outer holokarst dominated by Upper Cretaceous and partly Eocene limestone (Gnjato, R., 1991). Furthermore, the geological content of the outer field are Eocene flysch, rock conglomerate, and

breccia, whereas alluvial sediments are located in the field bottoms. The Eastern Herzegovina karst fields range in the Dinarides direction and lean towards the Adriatic basin, and a special characteristic are the underground karst hydrographic features.

Fatničko polje is located in the municipality of Bileća, 3 Kk southeast of Dabarsko polje. It covers 9.6 km^2 at the altitude of 400-500 m, and is situated among the mountains of Kosmatuša, Brusnika, Kuka, Oblog Brdo and Gruba Glava. Mt. Humac (510 m) makes the separation line into Gornje and Donje Polje. At the northeast rim of Gornje polje, there are periodical springs called Obod and Baba jama, along with a number of small constant springs. The Fatnička River flows down the field and heavy fall rain fills it with abundant water. During the flood season (spring and fall), most of the Fatničko polje becomes a periodical lake (Gnjato, O., 2004).

Sample collection

Fishnets were used for sampling fish in the Fatničko polje site after the flooding on the 26th and 27th March 2013. The sample consisted of 47 specimens.

Morphometric features

We determined the values of basic morphometric features, i.e. total and standard length, as well as the mass and Fulton coefficient. In order to estimate the mass, technical scales were used, and the standard and total length was measured by an ichthyometer.

Hematological analysis

The erythrocyte lineage parameters included the number of erythrocytes (RBC), hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Blood sampling was performed by heart puncture with a sharp and sterile needle (1.0 to 1.2 mm), under sterile conditions. Further analyses were performed with native blood without anticoagulant.

The number of erythrocytes was determined in a Neubauer chamber (hemocytometer) according to method of Kekić and Ivanc (1982). Hb was estimated by Drabkin hemoglobin cyanide method (Blaxhall and Daisly, 1973), and PCV was established by using microhematocritic centrifuges. Hematological indices (MCV, MCH and MCHC) were calculated from the values of packed cell volume, number of erythrocytes and hemoglobin concentration.

Statistical analysis

Data processing involved the use of both descriptive and analytical statistics and was performed via SPSS 16 and Excel 2007 statistical programs.

RESULTS

The study was performed on 47 specimens of *Delminichthys ghetaldii*, 7 males and 40 females, caught in the region of Fatničko polje after it was flooded in March 2013.

Mass and morphometric parameters

Statistical analysis of the morphometric parameters, mass and Fulton coefficient of the total sample and of males and females are presented in Table 1.

The Shapiro-Wilk test was used to estimate the normality of distribution of values of body mass, total and standard body length and Fulton coefficient. It indicated that all these parameters had normal type of distribution of their values.

Comparison of morphometric features of *Delminichthys ghetaldii* by gender indicated that females had larger total ($p = 0.015$) and standard length ($p = 0.029$), whereas the males had a somewhat larger Fulton coefficient.

Hematological parameters

All the analyzed erythrocyte parameters are given in Table 2.

Table 1. Total and standard length, mass and Fulton coefficient of *Delminichthys ghetaldii* from Fatnica field. Values of total sample and of males and females

	Statistical parameter		Total length cm	Standard length cm	Mass g	Fulton coefficient
Total	Mean		14.13	11.98	25.49	1.45
	Standard deviation		1.36	1.22	7.35	0.16
	Minimum		11.60	9.80	14.37	1.16
	Maximum		17.80	14.90	47.76	1.82
	95% Confidence Interval for Mean	Lower Bound	13.74	11.62	23.33	1.41
		Upper Bound	14.53	12.34	27.65	1.50
	Coefficient of variation - %		9.6	10.2	28.8	10.9
Males	Mean		13.00*	10.96*	20.83	1.54
	Standard deviation		1.06	1.12	7.08	0.11
	Minimum		11.60	9.80	14.37	1.44
	Maximum		14.50	12.60	32.18	1.69
	95% Confidence Interval for Mean	Lower Bound	12.02	9.92	14.29	1.44
		Upper Bound	13.98	11.99	27.38	1.64
	Coefficient of variation - %		8.1	10.2	34.0	6.9
Females	Mean		14.33*	12.16*	26.31	1.44
	Standard deviation		1.31	1.16	7.17	0.16
	Minimum		11.70	9.80	14.58	1.16
	Maximum		17.80	14.90	47.76	1.82
	95% Confidence Interval for Mean	Lower Bound	13.91	11.79	24.01	1.39
		Upper Bound	14.75	12.53	28.60	1.49
	Coefficient of variation - %		9.2	9.6	27.3	11.3

An asterisk in superscript denotes groups with significantly different mean values of the given parameter ($p \leq 0.05$)

The Shapiro-Wilk test indicated that the estimated distributions of RBC, PCV and MCHC values significantly deviate from the symmetrical one (RBC, $p = 0.021$; PCV, $p = 0.004$ and MCHC, $p = 0.000$).

Regarding the distribution of RBC and PCV, only the skewness was outside the typical symmetrical distribution limits, but in MCHC, both skewness and kurtosis were outside the limits typical of normal distribution.

Student's t-test and Mann-Whitney test were used to evaluate the significance of differences between mean values of the target parameters of males and females. The first test was used for the parameters with symmetrical distribution, and the latter for nonsymmetrical distribution of values. The Student's t-test showed that the values of total length were significantly ($p = 0.015$) higher in females (14.33 cm) than in males (13 cm). Comparing the mean values of the standard body length of males (10.96 cm) and females (12.16 cm), it was found that the value was statistically higher in females ($p = 0.029$).

Since the mean values of RBC did not have a symmetrical distribution, the Mann-Whitney test was used to evaluate the significance of difference between mean values between males and females. It was shown that males had a significantly higher RBC value than females ($p = 0.010$).

Correlation of body features and hematological parameters

Pearson's correlation was performed to estimate the correlation between parameters and its strength (Ta-

Table 2. Hematological parameters of *Delminichthys ghetaldii* from Fatnica field. Values of total sample and of males and females.

	Statistical parameters		RBC x 10^{12}/l	Hb (g/l)	PCV (l/l)	MCV (fl)	MCH (pg)	MCHC (g/l eryt.)
Total	Mean		1.474	72.50	0.398	271.19	49.36	193.16
	Standard deviation		0.126	8.76	0.083	59.65	6.17	59.78
	Minimum		1.190	55.56	0.176	121.70	37.99	117.28
	Maximum		1.820	92.59	0.500	375.94	63.57	419.75
	95% Confidence Interval for Mean	Lower Bound	1.437	69.93	0.373	253.68	47.55	175.61
		Upper Bound	1.511	75.07	0.422	288.70	51.18	210.71
	Coefficient of variation		8.6	12.1	20.9	22.0	12.5	30.9
Males	Mean		1.593*	73.54	0.384	242.16	46.38	196.12
	Standard deviation		0.133	3.96	0.067	43.79	3.67	32.77
	Minimum		1.430	66.67	0.286	184.16	40.91	154.81
	Maximum		1.810	77.78	0.455	303.03	51.85	233.33
	95% Confidence Interval for Mean	Lower Bound	1.469	69.88	0.322	201.66	42.98	165.81
		Upper Bound	1.717	77.21	0.447	282.65	49.77	226.43
	Coefficient of variation		8.4	5.4	17.5	18.8	7.9	16.7
Females	Mean		1.454*	72.31	0.400	276.27	49.89	192.64
	Standard deviation		0.114	9.38	0.086	61.02	6.40	63.62
	Minimum		1.190	55.56	0.176	121.70	37.99	117.28
	Maximum		1.820	92.59	0.500	375.94	63.57	419.75
	95% Confidence Interval for Mean	Lower Bound	1.417	69.88	0.372	201.66	47.84	172.29
		Upper Bound	1.490	77.21	0.428	282.65	51.94	212.99
	Coefficient of variation		7.9	13.0	21.6	22.1	12.8	33.0

An asterisk in superscript denotes groups with significantly different mean values of the given parameter ($p \leq 0.05$)

Table 3. Pearson's correlation coefficients between different parameters of *Delminichthys ghetaldii* from Fatnica field. N= 47.

	RBC	PCV	Hb	MCV	MCH	MCHC
Total length	-0.372*	0.212	-0.055	0.354*	0.206	-0.155
Standard length	-0.376**	0.206	-0.049	0.348*	0.211	-0.142
Mass	-0.327*	0.357*	-0.109	0.475**	0.117	-0.309*
Fulton coefficient	0.1	0.320*	-0.211	0.267	-0.283	-0.405**

*Statistical significance is denoted with asterisks in superscript - * $p < 0.05$; ** $p < 0.01$.*

ble 3). The number of erythrocytes was in negative correlation ($p < 0.05$) to body mass and total and standard body length. However, MCV was in positive correlation to the same parameters ($p < 0.05$). MCHC was in negative correlation to the body mass ($p < 0.05$) and Fulton coefficient ($p < 0.01$). At the same time, the PCV value was in positive correlation to both these parameters ($p < 0.05$).

As there was a strong correlation between the hematological parameters on the one side, and body mass, length and Fulton coefficient on the other, multiple regression analysis was performed. In this way it was estimated which variables could be used as reliable predictors. A common idea is that the somatic characteristics of an individual affect their hematological parameters (Butler et al., 2006). However, the

opposite situation was found in several studies and it was shown that hematological variables largely affect metabolic processes, thus enabling the development of many body features (Rehulka and Adamec, 2004; Alegbeleye, 2005; Ivanc et al., 2013).

Bearing this in mind, hematological parameters were observed as independent variables and body features as dependent variables. The approach to regression analysis, where hematological parameters were independent variables and body features were dependent, was based on the existence of significant correlation among the variables. Given that RBC is in significant negative and MCV in significant positive correlation to the total body length, these hematological variables were used as predictors in a multiple regression. It turned out that the number of erythrocytes and MCV taken together can explain 21% of total length ($p = 0.006$).

However, taken separately these hematological variables are weaker predictors of total length. For RBC significance is $p = 0.042$, and for MCV $p = 0.062$. RBC is also in negative and MCV in positive correlation to the standard body length. The results of the multiple regressions are similar to those for total length, and indicate that 20% of standard length can be explained by RBC and MCV taken together. The results are statistically significant, $p = 0.007$. Separately, only the RBC coefficient of determination is significant – $p = 0.038$. RBC and MCHC are in significant negative, and PCV and MCV are in significant positive correlation to the body mass of *Delminichthys ghetaldii*.

These four hematological parameters taken together are responsible for 28.7% mass prediction of *Delminichthys ghetaldii* with a high significance ($p = 0.006$). The determination coefficient is $R^2 = 0.287$. Individually none of these hematological parameters has a significant influence. However, stepwise multiple regression reveals that MCV accounts for 22.6% of body mass with high statistical significance ($p = 0.001$). The results of the hematological research of *Delminichthys ghetaldii* and the statistical processing provide valuable information on the range of their physiological variation and their normal values. The correlation of the hematological and body variables, and the results of the multiple regression analysis, are the basis for predictions of dependent variables.

DISCUSSION

The body length values that were estimated in this research were larger than those provided by other authors were. Vuković and Ivanović (1971) stated that *Delminichthys ghetaldii* reaches the length of 12.5 cm, and Kottelat and Freyhof (2007) as well as Jelić et al. (2008) specified that the body length of this species could maximally reach 13 cm, while the mean values of both total and standard length determined in this research were larger. On the other hand, Sofradžija (2009) stated that *Delminichthys ghetaldii* reaches the length of 12-15 cm, which is in accordance with the results of this study. Fish that appear from the abyss during spring floods have a high proportion of spawners, which could explain their larger mass and length. In agreement with this, Aganović and Kapetanović (1967) wrote that *Delminichthys ghetaldii* from the Ljubomirska River grow larger as they age and that the maximum growth rate is reached in the third and fourth year. Furthermore, other papers state that individuals of *Delminichthys ghetaldii* from specific cave abysses are larger than individuals from others (Čučković, 1983).

There are no available data on hematological parameters, which makes our study even more important. Namely, this research provides both lower and upper limits of confidence intervals for mean values with 95% probability, and for parameters of erythrocyte lineage of this species in natural habitats, thus providing a relevant basis for the estimation of their intervals. Normality of distribution of values of a given physiological parameter is considered a essential characteristic (Conover, 1980; Sokal and Rohlf, 1995), and in this study it also proved to be valuable.

Hematological parameters of *Delminichthys ghetaldii* were compared with the data of other cyprinids species, especially from the Leuciscinae

subfamily. The RBC values of *Delminichthys ghetaldii* are lower than those of *Telestes metohiensis* from the Pribitul stream, another endemic fish from karst waters in the same region (Dekić et al., 2012). Likewise, *Aulopyge hugeli, Leuciscus tursky, Chondrostoma phoxinus, Paraphoxinus alepidotus* and *Phoxinus phoxinus* had larger mean values of RBC as well as range of individual values (Vuković and Znidaršić-Križek, 1969). However, *Squalius cephalus* inhabiting the rivers Krupica and Željeznica (Mitrašinović and Suljević, 2009) had lower RBC values than *Delminichthys ghetaldii* from this study. Also, *Delminichthys ghetaldii* had higher RBC values than those obtained for *Barbus balcanicus* from two streams, Suturlija and Jakotinska rijeka, where the mean values of RBC were 1.148 $x10^{12}$/l and 1.099 $x10^{12}$/l, respectively (Dekić et al., 2009).

We can conclude that RBC varies within the same species in certain physiological limits and can alter along with the physiological condition of the organism so that the data on physiological range are different (Dekić, 2010). Stojić (1996, 2007) has stated that in 1 L of fish blood, the average RBC is between 1.0 x 10^{12} and 3.2 x 10^{12}, but according to Bogut (2006), in fresh water fish RBC varies between 0.7 x 10^{12}/l and 3.5 x 10^{12}/l, and in carp species it is between 1.8 x 10^{12}/l and 2.2 x 10^{12}/l. On the other hand, Tripahti et al. (2004) found that in common carp the mean value of RBC is 1.81 $x10^{12}$/l, ranging from 1.69 $x10^{12}$/l to 1.91$x10^{12}$/l. As we compare the hemoglobin concentration of *Delminichthys ghetaldii* from our research with values found in other cyprinid species from the region, there is a similarity with *Barbus balcanicus* and *Squalius cephalus* (Ivanc and Dekić, 2012), but they are lower in comparison with *Telestes metohiensis* (Dekić et al., 2012).

Comparing our results on the hematology of *Delminichthys ghetaldii* with the hematological parameters of other cyprinid species, it is evident that MCV and MCH are closest to *Squalius cephalus* and *Barbus barbus* values (Ivanc and Dekić, 2012), but slightly different from those of *Telestes metohiensis* (Dekić et al., 2012). At the same time, the MCHC values are lower in comparison with those estimated in *Telestes metohiensis* (Dekić et al., 2012). When all of the parameters of erythrocyte lineage of *Delminichthys ghetaldii* caught in the region of Fatničko polje are taken in consideration, it can be concluded that there exist certain specificities characteristic to this species, because each species has its typical values for different hematological features.

CONCLUSIONS

In this study, the morphometric and hematological parameters of *Delminichthys ghetaldii* caught in the region of Fatničko polje were researched, and mean values and confidence interval of means with 95% probability were determined as the relevant estimation of normal values. The results of the research substantially contribute to the understanding of the hematological parameters of endemic species *Delminichthys ghetaldii* in its natural habitat. The evaluation of the hematological parameters will help in the early detection of environmental changes or appearance of disease.

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