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Electrophoretical Patterns of Hemoglobins and Hemoglobin Concentrations in the Adults Trouts *Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica* of Ohrid Lake

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Abstract: Hemoglobins of Ohrid Lake trouts *Salmo aphelius* Kottelat 1997, *Salmo balcanicus* and *Salmo letnica* have been analyzed in agarose gel electrophoresis. Electrophoretical patterns of these three species revealed the presence of multiple hemoglobins: three anodic fractions and two cathodic fractions. This study provides information on hemoglobin electrophoretical patterns of fish individuals of the length 24-51 cm and weight from 150-1150 g. The electrophoretic fractions migrated closely together with a relative mobility of anodic bands (fractions) varying between 0.324 and 0.418 and of cathodic bands varying between 0.479 and 0.691. No difference has been detected for hemoglobin components on agarose gel electrophoresis and adult individuals between these three examined groups of Ohrid Lake trouts. Hemoglobin concentrations in the adults of *Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica* were in the same levels with some other salmonids.

Key words: Hemoglobins, gel electrophoresis, species, cathodic fractions, Ohrid Lake, salmonids

INTRODUCTION

Salmonid hemolysates contain multiple Hemoglobins (Hbs) that can be separated into anodal (HbA) and cathodal (HbC) components by high pH starch gel electrophoresis (Fyhn and Withler, 1991; Hochachka and Somero, 2002). In addition, several salmonid species display ontogenetic variation in their Hb patterns. Ontogenetic variation in the Hb patterns may reflect genetic adaptations to cope with differences in oxygen transport requirements that occur throughout the life cycle of the fish (Brunori, 1975; Giles and Randall, 1980).

The biological significance of the Hb multiplicity is not evident but possibly heterogeneous Hbs with different functional properties have adaptive value in a variable environment (Brunori *et al*., 1973; Sauer and Harrington, 1988).

The studies of many diverse species of fish have demonstrated that electrophoretically distinct hemoglobin components of fish species usually exhibit different oxygen binding characteristics (Hashimoto and Matsuura, 1960; Giles and Randall, 1980).

Ohrid trout or in former systematic name *Salmo letnica* is considered as the most important autochthonous fish of Ohrid Lake, from scientific and commercial point of view. Four forms of this fish species exist and are endemic in Ohrid Lake. The various trout forms which have been suggested to be different species are distinguished by their breeding time and habitat by which they in practice are thought to be reproductively isolated from each other and by its osteological characters (Kottelat and Freyhof, 2007). Genetic data have not supported their distinction however (Susnik *et al*., 2007). According to catalogue of fishes (Froese and Pauly, 2007), IUCN Red List of Threatened Species (Crivelli, 2006) and Fishwise (Universal Fish Catalogue), these Ohrid trout forms are *Salmo aphelios* (the former *Salmo letnica aestivalis*, (Stefanovic 1948), *Salmo letnica* (the former *Salmo letnica typicus* or *Salmo typicus*, *Salmo balcanicus* (the former *Salmo letnica balcanicus* and *Salmo lumi* (the former *Salmo letnica lumi*).

It is already known the application of hemoglobin electrophoresis to taxonomic studies of fishes from the publication of Tsuyuki *et al*. (1966), Scholofeld and Westman. Information on blood biochemistry parameters of Ohrid trout without any subdivision in forms have been published by Aliko and Hamitaj, Kalamishi *et al*. (2006), Latifi *et al*. (2008), Dimco and Beqiraj. Data on some blood biochemistry parameters have also been published for *Salmo balcanicus*, *Salmo aphelios* (Beqiraj *et al*., 2011a) and *Salmo typicus* (Beqiraj *et al*., 2011b) all them considered as forms of Ohrid trout (*Salmo letnica*). The present study aims determination of electrophoretic characteristic of hemoglobins of *Salmo letnica*, *Salmo typicus* and *Salmo aphelios* in natural conditions as an

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attempt to provide information on characterising these forms of Ohrid trout and as a proceeding line of studies on morphometric and haematological parameters of Ohrid trout forms.

MATERIALS AND METHODS

Fish specimens have been collected from six sites in the Albanian side of Ohrid Lake from the fish catch of local fishermen, by using the gillnets. The samples included 76 fish specimens: 26 specimens of *Salmo balcanicus*, 25 specimens of *Salmo aphelios* and 25 specimens of *Salmo letnica*.

Due to the fact that differentiation of three species of Ohrid Lake is based on phenotypic characteristics, ecological differences and different spawning seasons (Sell and Spirkovski, 2004) the fish samples were taken during the respective spawning seasons: in December 2009 for *S. balcanicus* in March 2010 for *S. letnica* and in August 2010 for *S. aestivalis*.

Each specimen has been weighted and measured. The maximum total length has been measured after Anderson and Gutreuter (1983). The weight varied from 150-1150 g and the body length varied from 24-51 cm. These ranges of weight and size correspond to the age over than 3 years according to Rakaj.

Blood samples have been taken after anesthesia with MS 222; 0.1 g L^{-1} water (Handy and Depledge, 1999). Blood was collected from the caudal vein with 5 mL sterile syringes and kept at ice-cold tubes to which heparin (70 IU mL^{-1}) had been added (Ballarin *et al*., 2004).

A part of the blood sample was immediately analyzed for Hemoglobin level (Hb) in field condition. Total blood hemoglobin content has been determined using cyanmethemoglobin method, based on the oxidation of hemoglobin to cyanmethaemoglobin in the presence of potassium ferricianide and the subsequent absorbance reading at 540 nm (Alexander and Griffiths, 1993).

The other part of blood sample has been used for electrophoretic procedure. Blood was centrifuged in field condition and it was immediately stored on ice. Erythrocytes were washed three times with saline solution and centrifuged three times at 10000 RTM for 10 min.

The hemolysates were stored at 4°C and they were used within 24 h for agarose gel electrophoresis. Identification of all hemoglobin fractions were carried out by horizontal agarose-gel electrophoresis (agarose concentration 0.8 g dL^{-1}, gel alkaline buffer: pH 8.5±0.1; buffered strips: pH 9.2±0.2) with automatic electrophoresis Hyris/Hydrasis, Sebia and scanned using a HYRIS densitometer/scanner at 570 nm or with a yellow filter (at the Clinical Chemistry Laboratory of University Hospital Center Mother Teresa in Tirana).

RESULTS AND DISCUSSION

Mean values of hemoglobin concentration of *Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica*, standard deviations, maximal and minimal values, mean error (m) and mean difference (t_{mes}) values are tabulated below (Table 1). The recorded mean hemoglobin concentration of *Salmo letnica* (10.13±2,5 g dL^{-1}) resulted higher than mean values of two other species. These values present a high significance: p>0.999.

Hemoglobin concentration (g/dL) of *Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica* have approximate values with the others species of Salmo (Sadler *et al*., 2000).

Hemoglobins' electrophoresis of 76 trout specimens has been done by using Hydragel Hemoglobin (e) K20 procedure in agarose gel. Each sample was run in one track of a single gel. The 10 electrophoretic runs were made in total as a number of samples have run in duplicate. All electrophoregrams were interpreted visually and SEBIA's HYRIS densitometer was used for all densitometric evaluations to complement the visual data as appropriate.

The hemoglobin of all investigated fish specimens showed two groups of components: one migrated to the anode (called the group A) and the other to the cathode (called group C). The number of components in the A group and in the C group was three and two, respectively. Figure 1 gives a diagrammatic representation of the

Table 1: Hemoglobin concentration (g/dL) of *Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica*

Species	No.	Mean±SD	Max.	Min.	m	t_{mes}
S. balcanicus	26	9.336±2.45	12.59	3.55	0.390	23.9***
S. letnica	25	10.13±2.5	15.25	7.80	0.411	24.6***
S. aphelios	25	8.726±1.958	13.70	4.37	0.400	21.8***

p>0.99**, p>0.999***

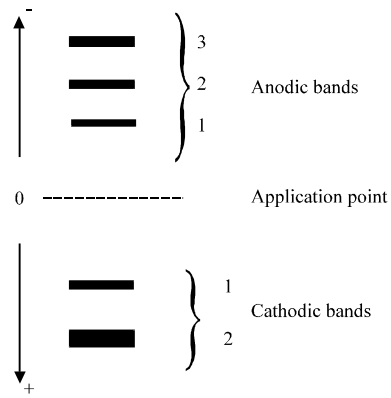

Fig. 1: Diagramatic represantation of agarose gel electrophoresis patterns of hemoglobins of the three species of Ohrid trouts

Table 2: Mean, SD, mean error (m) and mean difference (t_{mes}) of electrophoretical mobility (Rf) of each hemoglobin band

	Bands														
	Salmo balcanicus (n = 26)					*Salmo aphelios* (n = 25)					*Salmo letnica* (n = 25)				
	Anodic			Cathodic		Anodic			Cathodic		Anodic			Cathodic	
	1	2	3	1	2	1	2	3	1	2	1	2	3	1	2
Mean	0.332	0.363	0.413	0.479	0.635	0.3472	0.3796	0.4184	0.5316	0.6912	0.324	0.35	0.4	0.4871	0.6757
SD	0.0144	0.0097	0.095	0.0468	0.0414	0.00979	0.00734	0.00542	0.0385	0.0148	0.00975	0	0	0.059	0.0207
m	0.00322	0.0021	0.021	0.0104	0.0092	0.002	0.0015	0.0011	0.0078	0.003	0.0021	0	0	0.0128	0.0045
t_{mes}	103.1***	1728***	19.61***	46.05***	69.021***	173.6***	253.06***	380.3***	68.15***	230.4***	154.28***	E	E	38.05***	150.1***

Salmo balcanicus (p>0.95*, p>0.99**, p>0.999***); *Salmo aphelios* (p>0.95*, p>0.99**, p>0.999***); *Salmo letnica* (p>0.95*, p>0.99**, p>0.999***)

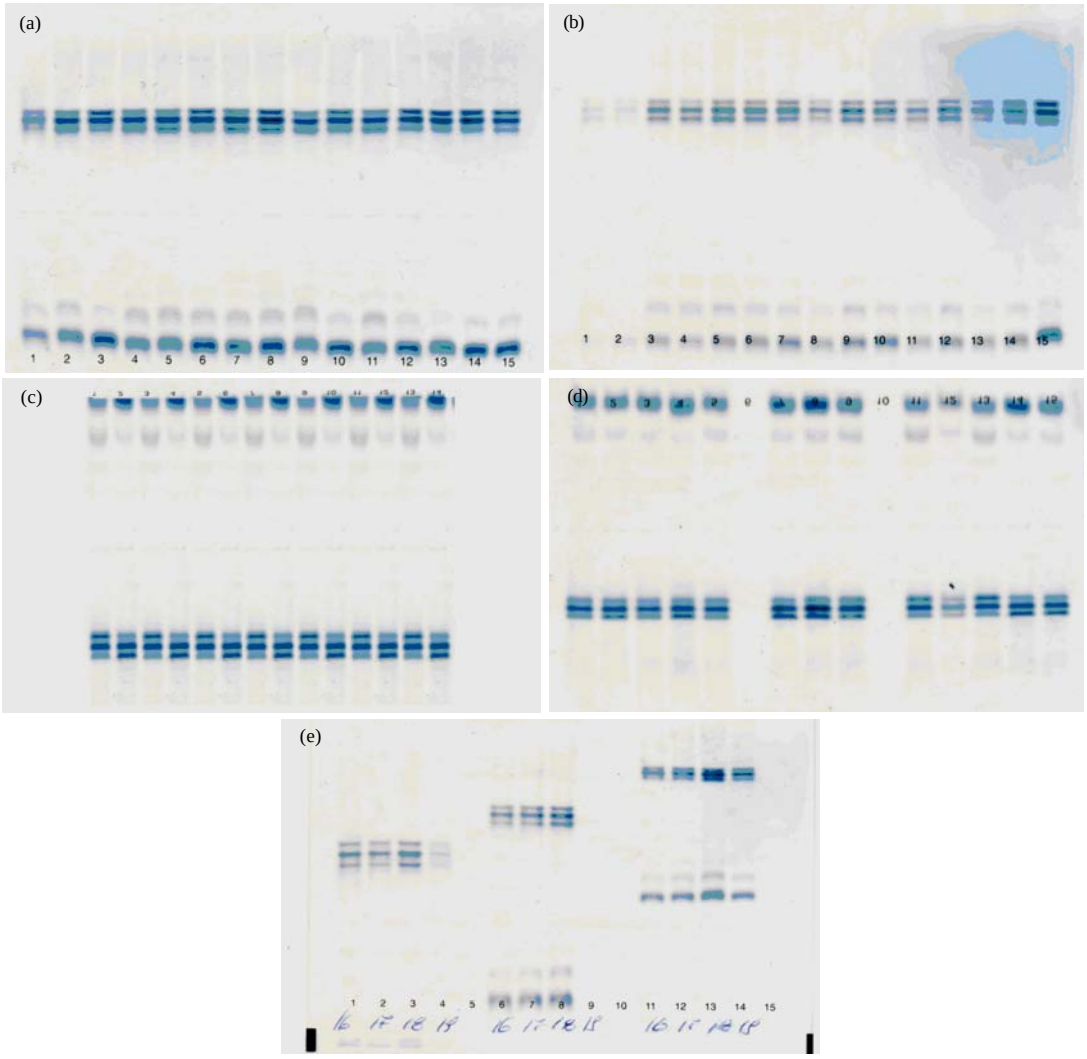

Fig. 2: Electrophoretical patterns of hemoglobins of a) *S. balcanicus*; b) *S. aphelios*; c) *S. letnica*; d) each of the three species in the same gel; e) with migration origin in the middle of gels and f) four samples in three different migration origins

hemoglobin patterns according to the number of components and direction of migration on *Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica*.

Electrophoretic patterns of hemoglobins of the three species are showed in Fig. 2, respectively *S. balcanicus* (Fig. 2a), *S. aphelios* (Fig. 2b) and *S. letnica* (Fig. 2c).

Figure 2d shows samples from each of the three species run in the same gel, aiming to assess and compare electrophoretical mobility of each band of hemoglobin. Figure 2e shows four samples run in different positions of the same gel in order to control that the expression of hemoglobin bands was not continuing after the length of these gels.

Densitometer scanning of stained electrophoregrames yielded electrophoretic mobility (Rf) of each individual hemoglobin band. The means and SD from densitometric data of each band in gels for each specimen of the three different species are tabulated (Table 2). A high significance ($p>0.999$) has been recorded for all evaluated Rf of 5 bands.

No difference has resulted in the number of hemoglobin bands between *Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica*. Hemolisates from all analyzed specimens had hemoglobin patterns with 5 bands: three anodic and two cathodic hemoglobin bands. The bands have migrated closely together with a relative mobility of anodic bands varying between 0.324 (the smallest Rf value recorded for *S. letnica*) and 0.418 (the highest Rf value recorded for *S. aphelios*) and of cathodic bands varying between 0.479 (the smallest Rf value recorded for *S. balcanicus*) and 0.691 (the highest Rf value recorded for *S. aphelios*).

Intraspecific variations in hemoglobin electrophoretical pattern have been found in some fish species and the study of gene frequencies for polymorphic hemoglobins has proved to be useful for taxonomic investigations. No difference has been found on hemoglobin components between the three examined species of Ohrid trouts, at least on agarose gel electrophoresis and for adult individuals. However, agarose gel elecrophoresis is considered efficient in determining hemoglobin components and intraspecific hemoglobin variations after Schlotfeld.

The hemoglobin in some fish species has been found to undergo an ontogenetic development which is restricted to a short larval or fetal phase or has proceeded during a great part of the life cycle (Yamanaka *et al*., 1967). Referring to the age-classes defined by Rakaj, the examined specimens of the Ohrid trouts in this study should be considered in adult phase, weighting 150-1150 g (average 382.8 g) and length 23-51 cm (average 33.6±6.06 cm). Smaller and larger sizes that might correspond to other life stages were not present in the samples. Consequently, it would not be able to state any opinion on ontogenic hemoglobin characteristics of these species. However, this study provides data on the electrophoretical hemoglobin patterns of fish size 150-1150 g which is the most common size of these trout species in the fish catch in the Albanian part of Ohrid Lake.

CONCLUSION

Mean hemoglobin concentration of *Salmo letnica* (10.13 ± 2.5 g dL^{-1}) is higher than mean values of two other species, *Salmo balcanicus* and *Salmo aphelius*. Hemoglobins of all analysed specimens of the Ohrid Lake trouts (*Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica*) showed one main electrophoretic pattern on high pH agarose gel. Its hemolisates had hemoglobin patterns with 5 bands. There is no difference in number of hemoglobin bands between three species of Ohrid trouts *Salmo balcanicus*, *Salmo aphelius* and *Salmo letnica*.

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